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BETEL, TOBACCO, AND CANCER OF THE MOUTH

C. S. MUIR AND R. KIRK*

From the Department of Pathology, University of Malaya in Singapore

Received for publication August 22, 1960

TOBACCO has been, for many years, under suspicion as a carcinogen. Pride of place has of late been accorded in the literature to the relationship between bronchial carcinoma and cigarette smoking, but in South India and in those parts of South-East Asia where persons of South Indian stock work, or have settled, oral cancer has long been a problem of importance. Essentially there are two types of oral cancer: that associated with the chewing of tobacco, usually admixed with some form of betel (*vide infra*), and that associated with smoked tobacco.

This paper is primarily, but not exclusively, concerned with the former type of tumour, and examines the nature of the betel quid, touches briefly on its complex chemistry and pharmacology, reviews the clinical and experimental evidence pointing to the presence of a carcinogenic agent, probably tobacco, in the quid, and reports experiments in which malignancy was induced in the ears of mice painted for a prolonged period with an aqueous extract of a typical Singapore betel quid.

The Quid: Composition, Chemistry, Pharmacology

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the leaf of the betel vine (*Piper betle*) on which catechu, an aqueous extract of the heart wood of the acaciae *Acacia catechu* or *Acacia suma* has been smeared. Spices such as cardamom, cloves and aniseed may be added for additional flavour (Sanghvi, Rao, and Khanolkar, 1955). This quid is inserted in the gingivo-buccal fold and chewed for hours (Shanta and Krishnamurthi, 1959).

In Thailand turmeric, the ground root of *Curcuma aromatica* is usually added to the chew (Ellis, 1921). The aboriginal Veddas of Ceylon prepare their slaked lime from the shells of snails (Spittel, 1924), and coral is not infrequently used in the Pacific Islands (Eisen, 1946).

While the method of preparation in Malaya and Singapore is essentially similar to that in India, the catechu is exhibited in a rather different form as " gambir " (*vide infra*).

The betel vine is cultivated, the leaves used for chewing being on the horizontal upper side branches ; the connoisseur's leaf being the largest. Plucking is done in the early morning, and the leaves are protected from the sun to preserve the aroma. The leaves are then bleached, the superior quality being very soft and coloured a uniform green yellow (Burkill, 1935*b*). This process heightens flavour, which is due to the presence of volatile oils. The chief of these is eugenol, an unsaturated aromatic phenol, usually very pale yellow in colour, which has a strong pungent odour reminiscent of cloves, and a pungent spicy taste. This substance has antiseptic and local anaesthetic properties (Weatherby and Haag, 1958). Chewing a betel leaf for five minutes leaves the mouth rather numb.

Terpenes are also present, these are pungent, and unpleasant if present to excess. Unusually large amounts of potassium nitrate, and small quantities of sugar, starch and tannin have been found (Mann and Patwardhan, 1916). The chewed leaf is a gentle stimulant and carminative, sweetening the breath (Burkill, 1935*b*).

The areca nut contains many alkaloids ; arecoline, arecaine, guvacine, arecolidine, guvacoline, *iso*-guvacine and choline (Henry, 1949). Arecoline is the only one of importance, the dried nut containing about 0·1 per cent. This alkaloid is cholinergic, exerting a sialogogue and diaphoretic action in normal dosage. Very large amounts depress the central nervous system. It may exert a deleterious effect on the dental enamel (Riker, 1958). Also present are tannin, the glycerides of lauric, oleic and myristic acids, and a little sugar. This nut is sometimes used internally by the Malays as a vermifuge and as a cure for diarrhoea (Burkill, 1935*a*).

By itself the areca nut is highly acid and astringent to the taste. The addition of lime not only neutralises this to a large extent, as can easily be demonstrated *in vitro*, but also promotes the appearance of a red dye.

From the shrub *Uncaria gambir* the " gĕtah gambir " of the Malay, the " katta kambu " of the Tamil, is extracted. The delicacy of flavour of this product depends upon its catechin content. The leaves are bound, steamed, and then small amounts of boiling water are allowed to trickle through. On cooling catechin crystallises out, leaving the more soluble, and bitter, catechu tannic acid in solution. Usually a little bran is added and the bran-catechin mixture made into cakes. In the production of gambier for tanning a crude process is used, extracting most of the catechu tannic acid (Burkill, 1935*c*).

Apart then from the tobacco nicotine which may be present in the quid, and which apparently has the same power to effect habituation as that from smoked

tobacco, the betel leaf-areca nut-gambir-lime mixture promotes intense salivation (Eisen, 1946), mild exhilaration, and to a certain extent, sleeplessness. The essential oils give a pleasurable tang and impart a subjective confidence in the the wholesomeness of the exhaled breath. However, like other habits of a similar nature, betel chewing seems to be an acquired taste, the first chew, whether tobacco is present or not, causing giddiness and nausea. It should also be noted that the colour red, such a prominent feature of the chewed quid, and of the expectorated mouth juices, connotes good luck to Indians, Chinese and Malays, as well as to other Asian peoples. Betel chewing is a poor man's luxury, as a made-up betel/tobacco quid costs 5 cents local currency (one penny sterling) : this is about the price of a cigarette which lasts for a much shorter time.

Tobacco is smoked in many parts of India in the form of " bidis ", a variety of cigarette in which dried powdered sun-dried tobacco (*Nicotiana tabacum* or *Nicotiana rusticum*) is wrapped in a variety of leaves, usually the dried leaf of the temburni (*Diospyros melanoxylon*), the whole being secured at one end by a thin string (Sanghvi *et al.*, 1955). These " bidis " are not generally smoked by the Chinese or Malays in Malaya or Singapore, but they are popular with locally domiciled Malayalees and Tamils of Social Class V. A similar type of smoke is very popular with the Malay, the " rokok daun " composed of a thin central core of Siamese tobacco around which is wrapped the leaf of the Nipah palm (*Nipah fruticans*).

The taxonomy of the plants mentioned above has been described by Ridley (1922–25).

Clinical Evidence

The main interest in betel chewing has, of course, always been directed to its possible relationship with oral cancer. Many of the early writers held there was no such relationship (Maxwell, 1924 ; Wells, 1925), whereas others (Fells, 1908 ; Bentall, 1908) did. Davidson (1923) and Spittel (1924) pointed out the possible significance of tobacco in the quid. Most of the confusion arose from a failure to distinguish between betel and betel/tobacco quids. Ellis (1921) held a survey of medical opinion in Siam, the consensus of which was that there was no such relationship. However, pursuing the matter further (Mendelson and Ellis, 1924), he showed that in the 24,340 males attending Government clinics in Bangkok in 1922–23 there were 43 cancers, 49 per cent of which were in the mouth, and concluded that betel/tobacco was carcinogenic. Others, such as Davis (1915) writing about Buyo cancer of the Philippines, held that lime was the active agent.

Clinical evidence has slowly accumulated over the years, successive investigations tending to be more accurate statistically.

Orr (1933 in his classical description of oral cancer in betel/tobacco chewers, examined in detail 100 cases of oral cancer. Of these, 2 were not chewers, 9 chewed occasionally, 24 chewed from three to five quids a day, 40 chewed more than this number, and 25 slept with a quid in the mouth. In a control group of persons with no oral cancer corresponding figures were 34, 31, 23, 10 and 2. Orr does not state how his control group was selected. He showed that over two-thirds of the cancers involved the site directly irritated. 34 per cent of the tumours were on the lower alveolus, or between the alveolus and the cheek,

33 per cent involved the cheek alone, 15 per cent the tongue and the floor of the mouth, 10 per cent the upper jaw and palate, and 8 per cent the lips. He remarked that the lip tumours seemed to begin just inside the angle of the mouth and wondered if this might be due to the habit of squirting juice out of the corner of the mouth.

Eisen (1946) described betel chewing in the Southwest Pacific Islands. Here, as well as the areca nut, the leaves and pods of the *piper betle* are used. The lime is obtained from sea-shells or from coral. Tobacco is not used, and cancer of the mouth is virtually unknown : one case in 8000 adults admitted to a New Guinea hospital. The teeth of these chewers, although stained red, were in good condition, unlike those of the betel/tobacco chewers of Madras (Shanta and Krishnamurthi, 1959).

That this susceptibility to oral cancer following the chewing of tobacco is environmental rather than racial is adduced from the evidence of Friedell and Rosenthal (1941), who described 8 cases of oral cancer in white American males who habitually chewed tobacco *per se*. These tumours arose at the point where the quid was usually placed.

Khanolkar and Suryabai (1945) describe an unusual cancer of the lip associated with the use of unsmoked tobacco. This they found particularly prevalent in Bihar, in north-east India, accounting for a minimum of 12·6 per cent of oral carcinomata biopsied. They noted that patients with this cancer sought treatment at such a late date that biopsy was often considered unnecessary. This undue prevalence they considered was due to the use of " khaini ", a powdered admixture of dried tobacco leaf and lime. A pinch of the mixture is deposited in the groove between the front lip and the teeth, being left there, until after dilution by saliva, it is swallowed. This process is carried out at frequent intervals throughout the day.

Sanghvi *et al.* (1955) undertook a statistical survey of 1460 patients referred to the Tata Memorial Hospital, Bombay, in 1952–54. Patients referred to this hospital in whom no cancer was detected formed a control group. Patients were asked whether they smoked " bidis " or chewed betel. It was shown that chewing of betel/tobacco was associated with cancer of the oral cavity ; chewing and smoking with tumours of the hypopharynx and base of the tongue ; smoking alone with cancers of the oropharynx, notably the tonsil, and the oesophagus.

Shanta and Krishnamurthi (1959) reviewed the 347 oral cancers seen in one year at the Cancer Institute, Madras. 71 per cent of all oral cancers (26·45 per cent of all malignancies) arose from the buccal mucosa, and 22 per cent (8 per cent cent of all malignancies) from the lingual mucosa. The incidence of cheek cancer was higher in the male ; environment, religion, anaemia, syphilis, tuberculosis, diabetes, hypertension, virus diseases and achlorhydria were not of significance. The habit of chewing tobacco, betel leaf and areca nut was highly significant. 85 per cent of those with a buccal cancer chewed all three, while in the non-cancerous control group the figure was 12·5 per cent. Only 8·7 per cent. of those with a cancer chewed betel nut and lime alone ; of the control group, 51·8 per cent. Smoking did not appear to be of importance, but gross dental sepsis was considered to be the main factor in the higher incidence of these tumours in the labouring, when compared with the lower middle class.

In carcinomas of the anterior two-thirds of the tongue the same factors held good, although tobacco smoking appeared to play a dominant role in cancers of

the posterior third. Sanghvi *et al.* (1955) attributed the high incidence of posterior third tumours to the combined habit of smoking " bidis " and chewing tobacco.

Another type of cancer associated with smoking is that of the hard palate. This is for the most part confined to Vizagapatam, and the outlying districts of Andhra Province, which is situated in the mid-eastern part of India. This cancer is almost certainly due to the smoking of " chutta ", a local type of cigar made by rolling dried tobacco leaf, which is then tied at the end with a thin piece of string (Khanolkar and Suryabai, 1945). Such a primitive device is difficult to smoke as the smoke is not easy to draw, the core being very irregular. As the lighted end goes out easily it is customary to keep it inside the mouth to promote combustion. For some reason it is only the women who use this " adda poga ", or reverse smoking, men from the same district, and of the same social standing, smoking the chutta in the orthodox manner (Reddy, Reddy and Rao, 1960).

Experimental Evidence

Woelfel, Spies and Cline (1941) tested the ether, alcohol and unsaponifiable fractions of areca nuts on mice, but failed to evoke tumours. While prolonged subcutaneous injection of tannic acid (tannin, gallotannin) has been shown to produce liver cirrhosis and eventually liver neoplasms, this substance did not evoke local tumours (Korpassy and Mosonyi, 1950). Although tannin is present in areca nut, and both catechu and catechu tannic acid may both be classed as tannins, their chemistry is so complex (Nierenstein, 1948) that each substance would need to be tested for carcinogenicity. To the best of the authors' belief no work has been done on the other constituents of the betel quid apart from tobacco.

Roffo (1939*a*, 1939*b*) prepared several distillates of tobacco ; a watery extract (100 to 120° C.), a thicker liquid (120 to 350° C.), and the residue. These products were applied daily to the ears of three batches of 20 rabbits for a period of 10 months. The first distillate evoked no tumours, but 95 per cent of those painted with the second, and 70 per cent of those painted with the residue developed squamous carcinomata. Sugiura (1940) was unable to confirm these findings using comparable distillates. Roffo (1941) showed that nicotine alone was not a carcinogen for rabbits.

Extracts of sun-cured Indian chewing tobacco have been prepared by Mody and Ranadive (1959) and assessed for carcinogenicity by painting the interscapular skin and buccal mucosa of Strong (A) and Swiss mice. Some extracts were screened by the method of Suntzeff, Cowdry and Croninger (1955). Hyperplasia of the epithelium was noted, but this may have been due to irritation. After the first month of painting shell lime was added to several of the extracts used on the buccal mucosa, without apparent result. The insertion of tobacco quids into the cheek pouches of hamsters proved quite ineffective. 3 : 4 Benzopyrene used as a control carcinogen produced cancers on the skin, but the same chemical placed on the buccal mucosa had no effect, the authors suggesting, like Levy, Gorlin, and Gottsegen (1951) that the absence of sebaceous glands to act as a portal of entry, and the protective action of mucin were responsible. Advanced hyperplastic changes were seen within two months of the application of alkaloid containing extracts to the skin, when simultaneously painted with

croton oil, but a poor survival time did not permit further study. The effect of a single subcutaneous injection of extracts was also followed. One mouse, injected with a total extract, developed a transplantable palpable fibrosarcoma in the subcutis not far from the site of administration. Large numbers of apparently spontaneous tumours were seen in the animals used in these experiments, but they occurred with equal frequency in the control stock.

Johnstone and Plimmer (1959) in their exhaustive review of the chemical constituents of tobacco remark, "a large amount of research has been concentrated on the aromatic hydrocarbon content of tobacco smoke, whilst fresh and processed tobaccos have attracted specifically less attention". Nevertheless 3 : 4 benzopyrene has been identified in extracts of fresh and processed leaves (Bentley and Burgan, 1958). There is some evidence to suggest that this may be derived from the atmosphere (Campbell and Lindsey, 1956).

There is of course ample evidence to show that the tars produced by pyrolysis of tobacco contain carcinogens (Wynder, Graham, and Croninger, 1953). Reddy *et al.* (1960) describe experiments in which the effect of tobacco tar, produced by burning "chuttas" and drawing the smoke through acetone, was determined on mice. Painting of the backs of the mice with the tar alone, basal cell proliferation and hyperplasia of the sweat and sebaceous glands were seen. There was no evidence of any neoplastic change at the end of four months. When heat was applied to the skin of the mouse after painting with tar, early malignancy was seen by the third month, and invasion of the dermis by the fourth. A temperature of 58° C. was chosen as it had been shown, by thermocouple, that this was the palate temperature of the "chutta" smoker. Khanolkar and Suryabai (1945) using a long stem thermometer recorded a somewhat higher mean temperature of 65° C. Reddy *et al.* (1960) concluded that there was not only a shortening of the latent period when heat was used, but there was an increased tumour yield, and felt that these observations explained the higher incidence of cancer of the hard palate among "chutta" smoking women.

In view of the comparative paucity of published work, and that conflicting, it was decided to paint the ears of Swiss white mice with an extract as near to that present in the mouth of the betel/tobacco chewer as could be devised.

The paint was prepared daily just before use. Three betel vine leaves were placed on a bench, and the inner smeared with moist stone lime. The equivalent weight of dried lime was about 0·2 g. On this were placed shavings of betel nut, approximately 4·0 g., and about 0·5 g. of "gambir" together with about 1·0 g. of sun-dried tobacco imported from South India. (Indonesian tobaccos are sometimes used in Singapore.)

The leaves were then wrapped round these ingredients and the whole transferred to a brass mortar and pestle to be ground for 5 minutes. 2 ml. of water were added, mixed throughly with the ground material, and the resultant dark red mass squeezed by the fingers. The fluid so obtained, temperature 28° C., also dark red in colour, was then painted on the ears of the mice by a number 5 camel hair brush and allowed to dry. Drying took about 10 minutes. For the first few days the mice seemed rather irritated, but thereafter the act of painting did not seem to worry them, nor did they make attempts to clean the betel juice off.

Initially a pilot trial using 12 numbered Swiss white, brother-sister mated, mice was instituted. They were painted daily for two years. Special care was

taken to ensure that the mice had an adequate amount of Vitamin A in the diet, as it has been shown that, in the rat, lack of this substance may cause keratinisation of epithelial tissues, round cell infiltration and secondary inflammatory change (McCarrison, 1931). The mice were kept in a non-airconditioned room (average temperature 28° C. : average relative humidity 84 per cent).

No macroscopic lesions were seen until 22 months had elapsed. A left ear was noted to be thickened, hardened and partially ulcerated. Painting was stopped, and the ear biopsied. After healing, the wound broke down again six months later. Ulceration of the right ear occurred one month after the left. Sections revealed loss of a large part of the ear, the bare area being covered by a fibrin cap which contained moderate numbers of polymorphs. The remaining skin towards the base of the ear, on both inner and outer aspects, showed marked thickening, often focal in nature and numerous intra-epidermal keratin filled cysts. There was no evidence of malignancy and inflammatory changes were not prominent.

Similar changes were seen in a second mouse at much the same time, and in a third, one month after painting had ceased. Ulceration of the left ear continued slowly over three months, when the right ear showed similar changes.

A fourth mouse showed ulceration of the base of the right ear laterally 32 months after the trial started, i.e., 8 months after painting had stopped (Fig. 1). A regional lymph node was markedly enlarged on palpation.

Sections showed an invasive squamous carcinoma (Fig. 2) arising in a polypoid excrescence containing numerous keratin filled cysts, some of which communicated with the surface. Cell nests were prominent. Parts of the invading tumour had reached the perichondrium. The skin on either side of the tumour was thickened and showed marked appendage change. The lymph node was invaded by a well differentiated squamous carcinoma of the same general structure as the primary tumour (Fig. 3). There was some doubt as to whether this was a direct extension or a metastatic phenomenon as the lymph node was somewhat torn. The subcapsular sinus showed marked catarrh.

The other ear showed thickening of the epidermis and ulceration of the tip.

In a fifth mouse, six months after painting ceased, a large subcutaneous nodule was seen to grow rapidly, just to the right of the midline, some 1·5 cm. behind the ears, reaching in five weeks a diameter of 1·5 cm. The swelling became ulcerated and the mouse was killed. The tumour was almost globular in shape and had a well marked pseudo-capsule, which was significantly infiltrated with round cells. Histologically a basi-squamous carcinoma, there were, principally at the growing margin, numerous islets of dark staining basal cells intermingled with very small differentiated cell nests (Fig. 4). Large areas at the centre were mummified, infected and necrotic. Calcification was seen in several places. The ulcerated portion of the tumour was grossly infected and covered by a dense fibrin cap. At the margins the epidermis was thickened with numerous mitotic figures in the basal layer. Appendages were replaced by epithelial plugs, several of which showed signal nuclear atypia. Similar, but lesser, departures from normal were present in most of the contiguous epidermis, as well as in both ears.

In a sixth mouse a benign squamous papilloma was found between the right ear and the rear fold of the fore-limb. This was well differentiated. The skin on either side showed moderate thickening and some appendage change. At death, of the remaining six mice, two showed relatively minor subcutaneous

sepsis at the root of the ears, in two there was no demonstrable lesion, in the fifth there was a reticulum type sarcoma. The sixth was discarded in error.

Painting of a second batch of 41 mice was begun in April, 1958, and is still in progress. The first lesion, noted six months later, was a papilloma situated at the base of the left ear. It was removed surgically. The epithelial covering was thick, differentiated and hyperkeratotic, being considerably infolded to form large keratin filled cysts (Fig. 5). There was one small area of questionable break-through. The subjacent dermis was rather oedematous and was conspicuously infiltrated by acute and chronic inflammatory cells. Numerous small blood vessels were noted.

The second lesion appeared in another mouse 7 months later. The left ear began to ulcerate, and within 15 days the entire ear had vanished. Painting was stopped as soon as ulceration was noted and eventually the ear site healed, to break down again six months later when it rapidly extended on to the neck tissues. The mouse was killed. Although infection, ulceration, and extensive epidermal and appendage changes were noted there was no focus of unequivocal malignancy. Gross thickening of the interscapular skin was seen in one mouse, and minor degrees of ear ulceration in two others.

In all a further 10 mice in this group have died. One had multiple tail and lung abscesses, another a blood-stained ascites of unknown cause, a third a form of gross ataxia, again of unknown cause, a fourth a liver cell carcinoma. No obvious disease was seen in the remaining six.

EXPLANATION OF PLATES

FIG. 1.—Mouse ear showing malignant ulceration. This could not be distinguished by the naked eye from non-malignant ulceration. The black pigment at the ear tip is dried paint.

FIG. 2.—Part of a well differentiated invasive squamous epithelioma from the mouse in Fig. 1. Note the large number of keratin-filled cysts on either side of this portion of the tumour. H. & E. × 27.

FIG. 3.—Well differentiated secondary tumour growth in lymph node. Same mouse as Fig. 1 and 2. H. & E. × 90.

FIG. 4.—Basi-squamous carcinoma with clumps of basal type cells at the centre and cell-nest structures at the periphery. H. & E. × 90.

FIG. 5.—Squamous papilloma with a large keratin filled cyst. H. & E. × 18.

FIG. 6.—Section of normal mouse ear skin showing relatively undifferentiated two-layer epidermis. H. & E. × 370.

FIG. 7.—Mouse skin showing marked increase in thickness and in numbers of cell strata, with differentiation into basal, prickle cell, granular and keratin layers. H. & E. × 370.

FIG. 8.—Numerous small intra-epidermal cysts, filled with keratin, in hyperplastic skin at the base of an ear. The panniculus carnosus is seen at the bottom right. H. & E. × 110.

FIG. 9.—Grossly thickened and hyperplastic mouse skin. Note the large prickle cells. Mitoses are not prominent in this particular field. H. & E. × 370.

FIG. 10.—An epidermal process composed of well differentiated prickle cells, in close contact with, if not actually invading, the aural cartilage. H. & E. × 875.

FIG. 11.—The hyperplastic hyperkeratotic epithelium shows grossly shortened hair follicles whose mouths are filled by keratin plugs. H. & E. × 100.

FIG. 12.—Altered epidermal appendages showing remnants of both hair follicles (A) and sebaceous gland structures (B). A clump of basal cells is still present (C). Epidermal thickening and hyperplasia is obvious. H. & E. × 100.

FIG. 13.—Large hyperchromatic appendages which have undergone complete squamous metaplasia. Under higher magnification the basement membrane of the arrowed follicle seems to be absent. Such appendages may be difficult to distinguish from enlarged epidermal downgrowths. H. & E. × 100.

FIG. 14.—Cartilaginous metaplasia in fibrous tissue. The metaplastic cartilage is of the human type, not of the simple murine seen below. H. & E. × 100.

FIG. 15.—The hair follicles are shrunken into small sub-epithelial clubs or wedges. An infected keratin cyst is seen on the right. H. & E. × 100.

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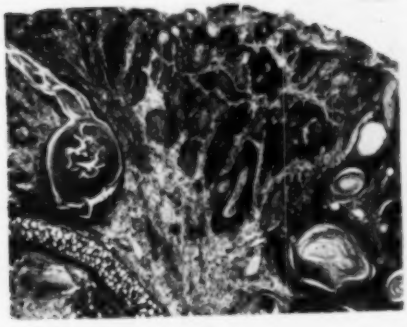

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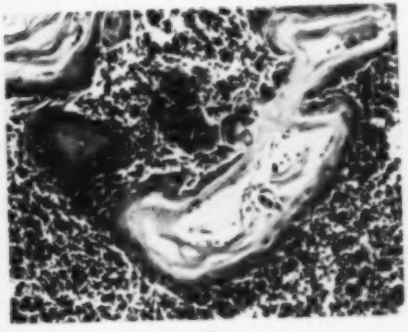

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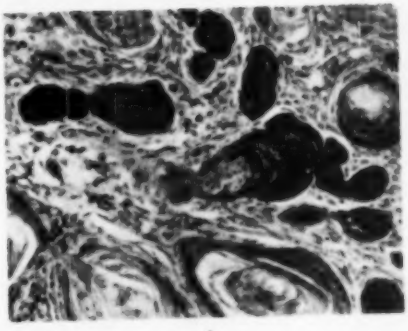

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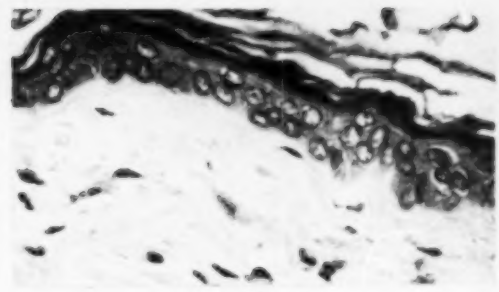

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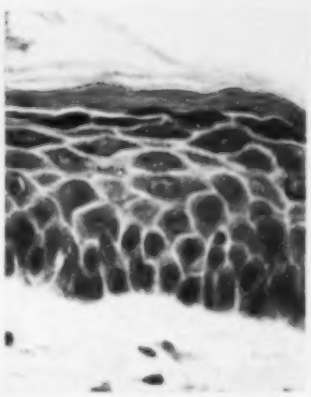

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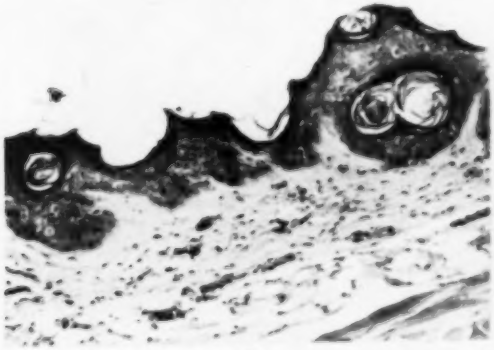

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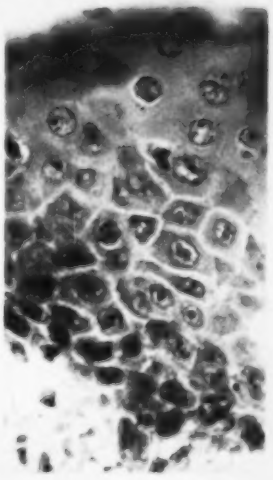

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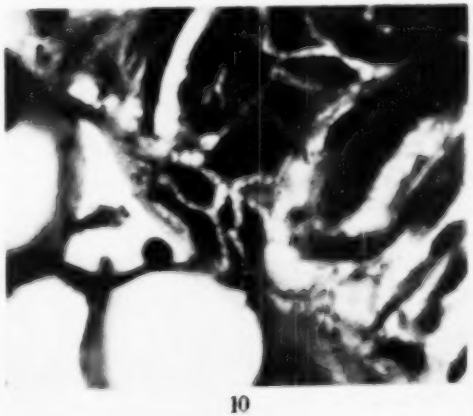

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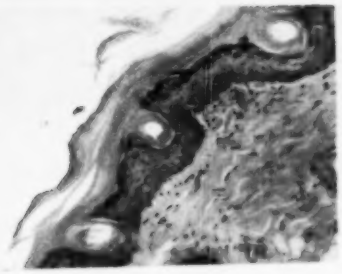

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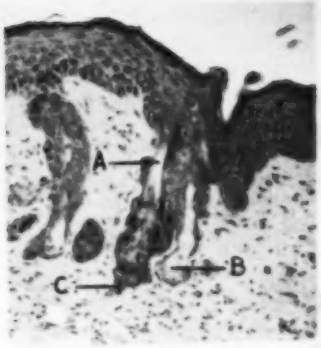

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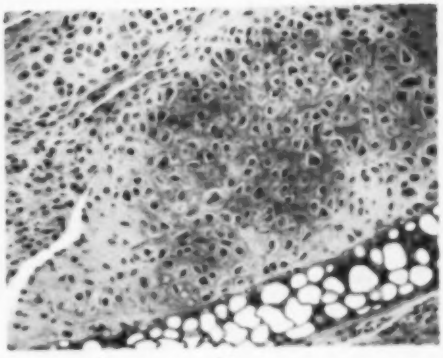

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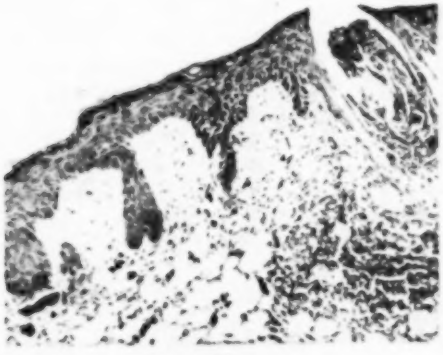

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The ears and surrounding skin of all the mice which have died, or been killed, have been examined histologically. Although we have, for financial reasons, been unable to start with a large number of animals and examine individuals at fixed intervals, nevertheless by study of the available sections a fairly comprehensive picture of the possible sequence of events can be arrived at.

Normal mouse skin is very simple (Glücksmann, 1945). Spinous and basal cells are contained in a single layer, which is covered by a thin keratin coat. In regions with a reduced number of hairs such as the ears, the epidermis is thicker and layering more obvious (Fig. 6) but nowhere does it approach the human type of epidermal covering.

The earliest change occurred in broad foci. The basal cells came to occupy the entire basement membrane ; a concomitant increase in the number of spinous cells gave rise to a definite prickle cell layer which differentiated into sizeable granular and keratin strata (Fig. 7). Irregular maturation of the keratin layer was seen, characterised by the presence of large cuboidal nucleated cells with an intensely eosinophilic cytoplasm. Elsewhere keratin was present to excess, often coated with particles of paint. Small intra-epidermal keratin-filled cysts were not uncommon (Fig. 8).

As well as this change into the human type of epidermis, there were focal areas of yet further hyperplasia. Here the prickle cell layer was much thicker, the spinous cells larger, the basal cells stained more deeply, and mitoses were present in increased numbers (Fig. 9). From such areas, epidermal downgrowths, often apparently without basement membrane, penetrated for varying distances into the dermis. These downgrowths remained very well differentiated, and were considered benign, although one such was seen to be in close contact with, if not actually invading the aural cartilage (Fig. 10).

Conspicuous epidermal appendage change was seen in about 65 per cent of the mice. Hair follicles seemed shortened, their mouths filled by keratin plugs (Fig. 11). Sebaceous glands showed squamous metaplasia, many retaining a thin basal cell layer on the outside (Fig. 12), while in a few this seemed to be absent (Fig. 13). Many of the appendages were scarcely recognisable as such, and were it not for the persistence of a central structure derived from the lumen of the original hair follicle, containing a small piece of keratin (Fig. 12), or the presence of clumps of large granular sebaceous cells (Fig. 12), would have been indistinguishable from enlarged epidermal downgrowths (Fig. 13).

Ulceration was common, invariably beginning in areas of hyperplastic epithelium. Many of these ulcers seemed to heal rapidly, dense collagen formation with parallel rows of fibrocytes beneath an intact epithelium being a frequent finding. In one mouse cartilaginous metaphasia was induced in new connective tissue laid down close to ear cartilage, this new cartilage being of a human rather than murine type (Fig. 14). Pre-existing collagen appeared normal.

Mast cells were present in increased numbers beneath many, but not all, of the areas of epidermal thickening, and were seen in very large numbers beneath the epidermis bordering on the malignant tumours.

Infection was very common, varying considerably in degree. Such areas were often covered by a thick infected crust, and the dermis seemed to contain large numbers of pigment-containing macrophages, the origin of the pigment being obscure.

In the control mice there were two spontaneous malignancies, one hepatic, the other probably uterine or ovarian, this latter ulcerating through the hind quarters.

DISCUSSION

It is difficult to evaluate how much of the change in the ears and surrounding skin was due to irritation by non-carcinogens, and how much to the carcinogen itself. Orr (1938) painted mice with benzene, a moderately powerful irritant, and observed, within 6 weeks, epithelial hyperplasia and some depilation. The hyperplasia was of the same nature as that seen with known carcinogens, but the number of cell layers was not so great. Many of the hair follicles were enlarged and hyperplastic with swollen internal root sheaths and enlarged hyperchromatic bulbs. There was never the appearance, such as is seen after one week of methylcholanthrene painting, where almost every hair follicle is shrunken into a small sub-epithelial solid wedge. This latter picture was not infrequently seen in our mice (Fig. 15), and indeed, most of the changes in our animals, if observed in a mouse painted with a known carcinogen, would be labelled as premalignant.

Dermal changes are equally difficult to assess. Gross infection was common. In mice with early epidermal changes, no alteration was seen in the collagen comparable to that described by Orr (1938) and others. Perhaps this may be ascribed to the relative weakness of the carcinogen.

The available clinical and statistical evidence all points to tobacco as the carcinogen in the betel/tobacco quid. Why our relatively crude experiments should have apparently succeeded in producing malignancies when the more sophisticated techniques of Mody and Ranadive (1959), using purer and stronger tobacco extracts, have failed, points, we feel, to the importance of co-carcinogenic factors. Which of the many components of the quid is of the greatest importance remains to be discovered, or, indeed, it may be that tobacco is the co-carcinogen for some other substance.

The arbitrary choice of the ear for painting may have been responsible in some measure for our success. Here the amount of underlying dermis is small, and is backed by a plate of relatively avascular cartilage. Any carcinogen passing through the epidermis was likely to remain there for some time before absorption, and not diffuse through to deeper tissues. The products of dermal degeneration, whether caused by irritation, or by the carcinogen, are also more likely to promote a newgrowth in the overlying dermis, as has been suggested by Orr (1938) and Marchant and Orr (1953). In this connection, it is noteworthy that although the tongue is as near the heat and smoke from a " chutta " smoked with the burning end in the mouth, as is the hard palate, Reddy and Rao (1957) in a series of 107 oral cancers in female smokers, found 68 palatal cancers for 14 lingual, i.e., 5 to 1. The dermis of the tongue has easy access to the underlying tissues ; that of the palate is backed by bone. Similar conditions obtain in the inner aspect of the labio-gingival fold (" khaini " cancers) and in the inner aspect of the buccal sulcus, but not, of course, on the cheek itself.

Although at present, as will be evident from the papers quoted, a problem of magnitude, it is our belief that the incidence of betel chewers cancer will fall, particularly in multiracial Singapore, as living and, probably more important, educational standards improve. In Malaya this decrease will be much slower as the isolation of South Indian labour on rubber estates and on Public Works

Department gangs, is responsible for the persistence of the chewing habit, as a closed community gives up such customs less readily.

SUMMARY

The geographical distribution, the history, and sociological importance, of betel chewing are briefly mentioned.

The chemistry and pharmacology of the contents of the quid are reviewed. The clinical, statistical, and experimental evidence pointing to tobacco as the carcinogen in the chew is examined. The failure of early writers to mention whether tobacco was present in the quid chewed is shown to have led to some confusion.

Experiments are described in which the ears of 53 Swiss white mice were painted daily for two years with an aqueous extract of a typical Singapore betel/tobacco quid.

In the first batch of twelve mice, all of which are now dead, two squamous cell carcinomas and a benign squamous papilloma appeared on or around the painted area, either during painting or after it had ceased. In a second batch of 41 similar mice, 30 of which are still alive, one active squamous papilloma has been noted to date.

The varying degrees of epidermal and dermal change seen in the ears of all the mice are recorded, and are found to be substantially the same in appearance as those caused by the carcinogenic cyclic hydrocarbons.

We wish to thank Professor J. W. Orr for useful advice and histological opinion, Mr. V. Nalpon for the photographs, our technical staff for the many sections, Mr. Rengasamy for care of the animals, and Mr. P. A. Samuel who typed the script.

REFERENCES

Bentall, W. G.—(1908) *Brit. med. J.*, ii, 1428.

Bentley, H. R. and Burgan, J. G.—(1958) *Analyst*, **83**, 442.

Burkhill, L. H.—(1935*a*) 'A Dictionary of the Economic Products of the Malay Peninsula', London (Crown Agents for the Colonies), Vol. I, p. 223.—(1935*b*) *Ibid.*, Vol. II, p. 1737.—(1935*c*) *Ibid.*, Vol. II, p. 2198.

Campbell, J. M. and Lindsey, A. J.—(1956) *Brit. J. Cancer*, **10**, 649.

Davidson, J.—(1923) *Brit. med. J.*, ii, 733.

Davis, G. G.—(1915) *J. Amer. med. Ass.*, **64**, 711.

Eisen, M. J.—(1946) *Cancer Res.*, **6**, 139.

Ellis, A. G.—(1921) *Arch. intern. Med.*, **28**, 252.

Fells, A.—(1908) *Brit. med. J.*, i, 1357.

Friedell, H. L. and Rosenthal, L. M.—(1941) *J. Amer. med. Ass.*, **116**, 2130.

Glücksmann, A.—(1945) *Cancer Res.*, **5**, 385.

Henry, T. A.—(1949) 'The Plant Alkaloids'. 4th Ed. London (Churchill), p. 8.

Johnstone, R. A. W. and Plimmer, J. R.—(1959) *Chem. Rev.*, **59**, 885.

Khanolkar, V. R. and Suryabai, B.—(1945) *Arch. Path.*, **40**, 351.

Korpassy, B. and Mosonyi, M.—(1950) *Brit. J. Cancer*, **4**, 411.

Levy, B. M., Gorlin, R. and Gottsegen, R.—(1951) *J. nat. Cancer Inst.*, **12**, 275.

Mann, and Patwardhan—(1916) *Mem. Dep. Agric. India, Bact.*, **4**, 321. Quoted by Burkhill (1935*b*).

MARCHANT, J. AND ORR, J. W.—(1953) *Brit. J. Cancer*, **7**, 329.
MAXWELL, J. L.—(1924) *Brit. med. J.*, i, 729.
MCCARRISON, R.—(1931) *Ibid.*, i, 966.
MENDELSON, R. W. AND ELLIS, A. G.—(1924) *J. trop. Med. (Hyg.)*, **27**, 274.
MODY, J. K. AND RANADIVE, K. J.—(1959) *Indian J. med. Sci.*, **13**, 1023.
NIERENSTEIN, M.—(1948) in Allen's *Commercial Organic Analysis*. 5th Ed. Philadelphia (Blakiston), p. 24.
ORR, I. M.—(1933) *Lancet*, ii, 575.
ORR, J. W.—(1938) *J. Path. Bact.*, **46**, 495.
REDDY, D. G. AND RAO, V. K.—(1957) *Indian J. med. Sci.*, **11**, 791.
Idem, REDDY, D. B. AND RAO, P. R.—(1960) *Cancer*, **13**, 263.
RIDLEY, H. N.—(1922–25) 'The Flora of the Malay Peninsula'. (5 Vols.) London (Reeve).
RIKER, W. F., JR.—(1958) in 'Pharmacology in Medicine' Edited by Drill, V. A., 2nd Edn. New York (McGraw-Hill), p. 367.
ROFFO, A. H.—(1939*a*) *Dtsch. med. Wschr.*, **63**, 1267.—(1939*b*) *Ibid.*, **65**, 963.—(1941) *Schweiz med. Wschr.*, **71**, 549.
SANGHVI, L. D., RAO, K. C. M. AND KHANOLKAR, V. R.—(1955) *Brit. med. J.*, i, 1111.
SHANTA, V. AND KRISHNAMURTHI, S.—(1959) *Brit. J. Cancer*, **13**, 381.
SPITTEL, R. L.—(1924) *Brit. med. J.*, i, 158.
SUGIURA, K.—(1940) *Amer. J. Cancer*, **38**, 41.
SUNTZEFF, V., COWDRY, E. V. AND CRONINGER, A.—(1955) *Cancer Res.*, **15**, 637.
WEATHERBY, J. H. AND HAAG, H. B.—(1958) in 'Pharmacology in Medicine'. Edited by Drill, V. A., 2nd Ed. New York (McGraw-Hill), p. 108.
WELLS, C. R.—(1925) *U.S. nav. med. Bull.*, **22**, 437.
WOELFEL, W. C., SPIES, J. W. AND CLINE, J. K.—(1941) *Cancer Res.*, **1**, 748.
WYNDER, E. L., GRAHAM, A. E. AND CRONINGER, A. B.—(1953) *Ibid.*, **13**, 855.

HISTOLOGICAL TYPES IN A LUNG CANCER MATERIAL IN VENICE

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In a series of countries a new lung cancer situation has been under progressive development in the present century. The new situation is characterized by an increased occurrence of lung cancer, especially in males in urban areas. The increase is mainly caused by carcinomas histologically designated as epidermoid (squamous cell) and small-cell anaplastic ("oat"-cell) carcinomas, whereas the other types of primary epithelial lung tumours seem to show a more constant and equal occurrence in males and females, in urban and rural areas, in all larger occupational categories as well as in smokers and non-smokers (Kreyberg, 1959).

Even if increased cigarette consumption is accepted as a major cause of this new situation, other factors, suspected or unsuspected, are probably involved and an international survey of the lung cancer development in different countries, with different habits and living conditions, is highly desirable, and the World Health Organization in its Technical Report No. 192 (1960) has recommended that such studies be made.

As part of such a programme the Director of the WHO International Reference Centre for Histological Definition of Lung Tumours (Professor L. Kreyberg) approached the Director of the pathological service of the Ospedali Civili Riuniti in Venice (Professor E. Ferrari) with a view to obtaining a preliminary orientation from a site exhibiting some peculiar conditions.

Venice is a town without automobiles. Tar and asphalt are used as street covers in negligible amounts, heavy industry is absent and no coal smoke pollutes the air.

On the other hand, Bastai and Pescetti (1959) have confirmed the statement of Fabris (1937) that in Venice lung cancer is just as prevalent, if not more so, as in other Italian cities.

Ferrari has from his autopsy material found that in males two out of every seven cases of malignant tumours are lung cancers, and that lung cancer is twice as frequent as stomach carcinoma, the second in numerical importance.

In the Venice autopsy material the sex ratio for lung cancer as a mean of the years 1951–59 is 10 males to 1 female. The corresponding sex ratio for the total autopsy material is 1·4:1.

All these figures substantiate the claim that the lung cancer rate in Venice is fairly but not excessively high.

In his "Congress Lecture" Kreyberg (1959) developed the thesis that the ratio Group I tumours (epidermoid and small cell anaplastic ("oat"-cell) carcinomas) to Group II tumours (adenocarcinomas, bronchiolo-alveolar carcinomas, carcinoids ("adenomas") and mucous gland ("salivary gland") tumours) is an indicator of the development of the new lung cancer situation, and that this ratio could be used as a measure of the total incidence of lung cancer in areas where

incomplete mortality and morbidity statistics only are available, and it was proposed to plan an international geographical study of lung cancer on this basis.

The World Health Organization has accepted these premises as a working hypothesis.

A study of this special problem therefore was included in our survey.

For the period 1956 to 1960 Ferrari was able to collect 78 cases for histological typing, and this typing has now been completed according to the nomenclature recommended for trial by an Expert Committee under the auspices of WHO.

The result of the typing is shown in Table I.

TABLE I.—*Distribution of Histological Types in Venice*

	Type							
	Group I		Group II			Others		
Sex	E	O	A	B	C	U	M	Total
Males	33	12	13	1	0	10	2	71
Females	0	2	3	0	0	1	1	7
Total	33	14	16	1	0	11	3	78

Ratio Group I : Group II.
Males 45 : 14 3·2 : 1.

E Epidermoid carcinoma.
O Small (" oat ") cell carcinoma.
A Adenocarcinoma.
B Brochiolo-alveolar carcinoma.
C Carcinoids.
U Anaplastic and uncertain types.
M Combined epidermoid adenocarcinoma.

Even if the material is limited it shows some remarkable features.

The first is a ratio Group I: Group II tumours of 3·2:1 in males. The corresponding ratio for Norway, a country with a known low incidence, is 3·4:1, as the mean for the last 12 years. According to the thesis of Kreyberg this would indicate a rather low total incidence of lung cancer also in Venice, a conclusion at evident variance with the frequency figures quoted above.

One explanation of the discrepancy could be that the Group II tumours in Venice had increased parallel with the Group I tumours.

Such an explanation would mean that the agent causing the rise in lung cancer in Venice would differ qualitatively from that operating in several other countries examined (Great Britain, Finland, Norway), or the population would differ in its response.

Another explanation could be a difference in the source of the materials studied, and such a difference actually exists. In the Venice material 67 out of 78 are autopsy cases, whereas in the Norwegian material there are only 36 out of 600. An examination of the male Norwegian autopsy cases reveals that here the ratio is only 1·8:1.

A second observation also indicates a fundamental difference in the two materials. Table II shows the age distribution of the Italian and the Norwegian cases.

In this table the Norwegian material does not include the carcinoids, the mucous gland tumours and the bronchiolo-alveolar cell carcinomas, because these

TABLE II.—*Age Distribution in Males*

Tumour groups	Age groups (years) −39	−49	−59	−69	70+	Total
			Venice			
Group I	1	4	11	20	9	45
Group II	—	1	3	9	1	14
Total	1	5	14	29	10	59
			Norway			
Group I	4	56	186	135	22	403
Group II	3	9	27	20	8	67
Total	7	65	213	155	30	740

tumours are nearly non-existent in the Italian material and they often occur in the lower age groups. Whereas two-thirds of the Italian cases are aged 60 years or more, the figure is a little more than one-third for the Norwegian, in spite of the fact that the population at large shows a relatively higher representation of old people in Norway than in Italy.

Finally, in Venice there is no thoracic surgical unit, and accordingly a certain number of younger lung cancer patients go to Padova for special treatment. A certain selection of prognostically more favourable cases, probably including a number of epidermoid carcinomas, may therefore have taken place, making the Venice figures qualitatively less representative.

This preliminary study of histological types occurring in the Venice autopsy material points to some factors to be considered when comparative studies are made.

The place of the lung carcinoma as the number one among the malignant tumours in males in an aggregate hospital with no special thoracic unit strongly supports the conclusion that lung cancer in Venice truly is rather frequent.

If this conclusion is correct, the Venice material has shown an unexpectedly high number of Group II tumours, nearly exclusively consisting of adenocarcinomas. The absence of carcinoids, another peculiarity, may be incidental because of the limited number of cases. Nevertheless, the low ratio Group I:Group II tumours is remarkable and an explanation must be sought for, even if the ratio 3·2:1 should most correctly be compared to the Norwegian autopsy fraction with a ratio 1·8:1.

From a methodological point of view this small study underlines the necessity, when comparative studies of lung cancer types are made, to use materials of similar character.

From the factual side, the study indicates that a more extended future study of the Venice material is highly desirable, first to ascertain with greater certainty the true relative occurrence of the different histological types, and next, if the present findings are confirmed, to seek an explanation of the great number of adenocarcinomas as compared to the number of Group I tumours.

REFERENCES

BASTAI, P. AND PESCETTI, G.—(1959) *Minerva med., Torino*, **50** (1), 1.
FABRIS, A.—(1937) *Tumori*, **23**, 1.
KREYBERG, L.—(1959) *Acta Un. int. Cancr.*, **15**, 78.

THE HISTOLOGY OF CYLINDROMA OF MUCOUS GLAND ORIGIN

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IT is now generally agreed that classification of mucous gland tumours on histological grounds is a useful procedure since the various types of tumour bear differing prognoses. Though there is still lack of unanimity with regard to details of nomenclature, most pathologists and clinicians would probably agree to the recognition of the following neoplasms as distinct entities.

1. *Mixed tumours.*—These are the neoplasms traditionally described as "mixed salivary tumours". Typically, they consist of an epithelial component intimately associated with mucoid or myxochondroid intercellular material.
2. *Mucoepidermoid tumours.*—The neoplasms described by Stewart, Foote and Becker (1945), consisting of epidermoid and mucus-secreting elements.
3. *Cylindromas.*—Tumours of characteristic architecture, to be described in this paper.
4. *Glandular, squamous and other carcinomas.*—Frank carcinomas, mostly glandular, sometimes squamous and occasionally of other types, comparable in behaviour to carcinomas elsewhere.

These four groups of neoplasms constitute the great majority of all tumours of mucous glands and warrant separate recognition on grounds of structure and behaviour. In addition, there are the less common tumours such as those of the oxyphil, acinic cell and papillary cystadenoma type, and the mesenchymal tumours, which are not further considered here.

With regard to the behaviour of the commoner neoplasms, the mixed tumours are generally regarded as benign. However, they show a marked propensity for recurrence, due to factors such as the leaving behind of peripheral nodules of growth after enucleation, the implantation of neoplastic cells in the tumour bed and the presence of focal infiltrations (Patey and Thackray, 1958). Apart from recurrences due to such causes there is the question of whether mixed tumours can change in type to become carcinomatous. It is generally believed that such changes do occur in a certain proportion of cases (Foote and Frazell, 1953; Frazell, 1954; Patey and Thackray, 1958) but that on the whole they are infrequent.

The muco-epidermoid tumours, on the other hand, behave as malignant growths, both with regard to local invasion and metastatic deposits. The cylindromas show a similar natural history, for they too are locally invasive growths and are also capable of giving rise to metastases. Their rate of growth, however,

is generally rather slow and they tend towards multiple recurrences before finally metastasizing. The frank carcinomas behave more aggressively than the tumours just mentioned, running a course more like carcinoma elsewhere.

It is evident, therefore, that accurate histological diagnosis is essential for the prognosis and treatment of mucous gland tumours. The purpose of this paper, based on the study of 42 tumours, is to deal in detail with the histopathology of one type of these neoplasms, the cylindroma, and to indicate the wide variety of appearances which may be encountered.

Nomenclature

Billroth in 1859 gave the first account of a cylindroma. In his original case the neoplasm had invaded the orbit, probably from the mucous glands of the accessory nasal sinuses and he coined the name cylindroma because the epithelial elements of the growth were enclosed in well-defined " cylinders " of connective tissue. Malassez (1883) mentioned some earlier cases that appeared to fall into the same group. The term " basalioma " was first used by Krompecher (1908), who considered the tumour to be of analogous nature to the basal cell growths of the skin. Ahlbom (1935) and Ringertz (1938) also used the term basalioma. In the more recent American literature the tumour has generally been known as " adenoid cystic carcinoma ", a term apparently introduced by Ewing (Foote and Frazell, 1953), and series of tumours under this name have been reported by Spies (1930) and others. Spies emphasized that the tumour was a distinct entity and should not be confused with the adenoid cystic epithelioma of Brooks and Fordyce, and that it was different from adenocarcinoma. Adenocarcinoma of mixed tumour type has also been used as a designation (New and Childrey, 1931 ; Watson, 1935), and adenocarcinoma of cylindroma type (Dockerty and Mayo, 1942, 1943 ; Quattelbaum, Dockery and Mayo, 1946). The original term cylindroma has come back into favour recently, and Lennox (1960) comments " I can find no better name than ' cylindroma ' for the group, though few members contain any cylindrical structures ".

Tumours of cylindromatous type were at first regarded simply as variants of the " benign salivary gland tumour ", as it was often called, and were not considered to differ in any significant manner from the majority of such tumours. Nevertheless, even early reports had shown that these neoplasms were very apt to recur after local removal, and could even metastasize (Ribbert, 1907). On the other hand, some tumours which behaved in a malignant manner were thought of as malignant mixed tumours rather than the cylindromas they undoubtedly were, owing to inadequate appreciation of the structural distinctions between the two types of tumour. Later, however, the structural characteristics of the cylindroma came to be understood, and also a general appreciation of their clinical behaviour. Montanus (1938) and Mulligan (1943) review the earlier reports of metastasizing tumours of the salivary glands, many of which were undoubtedly cylindromas.

Distribution

It has been recognized for many years that as well as occurring in the major salivary glands tumours of cylindromatous type may also be found in the minor salivary glands within the oral cavity and in mucous glands elsewhere. Malassez

(1883) was one of the earliest authors to describe a tumour of the palate as cylindroma-like. A large number of reports of palatal mucous gland tumours have since appeared though the neoplasms are very often reported as " mixed " or " benign salivary " tumours. The literature of these earlier accounts may be found in Ringertz (1938). More recent reports of intra-oral salivary gland tumours have given some prominence to the question of histological types and most authors have stressed the necessity for distinguishing between " mixed " tumours and the other varieties of mucous gland neoplasms. Recent series are those of Rawson, Howard, Royster and Horn (1950), Russell (1955), Ranger, Thackray and Lucas (1956) and Harrison (1956, 1957). The intra-oral glands are the most frequent site of occurrence of cylindroma for though salivary gland tumours in general occur less commonly in the minor salivary glands the proportion of cylindromas occurring in those glands is about 15 per cent of all tumours (Ranger, Thackray and Lucas, 1956), whereas in the major salivary glands it is in the region of 4 per cent (Foote and Frazell, 1953). In the case of the major glands the parotid and submaxillary are chiefly affected, cylindromas of the sublingual gland being very rare. Intra-orally, the glands of the palate and the floor of the mouth are the main sites, about 70 per cent of all intra-oral cylindromas occurring in these two areas. The remaining tumours occur in the tongue, the lip, or elsewhere in the buccal mucosa.

Cylindroma also occurs in the respiratory tract with some frequency. In fact, in a large series of tumours from all sites there are likely to be more neoplasms from the respiratory tract, including the nose, naso-pharynx and sinuses, than from the salivary glands. However, it is only comparatively recently that cylindroma of the respiratory glands has been recognized as a distinct entity. Kramer and Som (1939) were the first to report a tumour of this type occurring in the bronchus, and they give the early literature for the tumour in other parts of the respiratory tract. Subsequently there have been numerous reports of such lesions, most authors giving prominence to the question of differentiating the various types of neoplasm that can arise from the respiratory glands. It is now generally agreed that a clear distinction should be made between cylindroma, bronchial adenoma of the carcinoid type and frank bronchial carcinoma (McDonald and Havens, 1948; Belsey and Valentine, 1951; Reid, 1952), though occasionally tumours occur which appear to share characteristics both of the solid adenoma and the cylindroma (Englebreth-Holm, 1944). In Enterline and Schoenberg's (1954) series, bronchial cylindromas proved ultimately fatal seven times as frequently as " carcinoid " adenomas, and also recurred seven times as frequently.

Cylindroma also occurs in the lachrymal gland, where it constitutes some 50 per cent of all tumours, the other 50 per cent being of the mixed type (Godtfredsen, 1948). Occasional breast tumours show the cylindromatous pattern (Stewart, 1946), as do a number of skin tumours deriving from the sweat glands (Lever, 1948).

Histology

General histological pattern.—The most commonly observed histological pattern in cylindroma is that shown in Fig. 1, the tumour consisting of irregularly shaped masses of cells in a rather scanty connective tissue stroma. Numerous cystic or alveolar spaces are present in the cell masses, giving rise to the cribriform effect which is a very characteristic feature of this neoplasm. However, not all

tumours show the cribriform pattern since the cystic spaces are sometimes absent. In such cases, the growth is of a solid type. Variations of the cystic or the solid pattern account for much of the variation to be observed in the general histological picture in cylindroma, though changes in the stroma also contribute in some cases.

The cystic pattern.—The cystic spaces in a cylindroma are not all formed in the same manner. In Fig. 2, for example, the space represents a duct, its wall consisting of a double row of cells arranged in a similar manner to those of a normal salivary gland duct. The cells of the inner row, which will be referred to as lumenal cells, are small, with oxyphilic cytoplasm and a vesicular nucleus which contains a prominent nucleolus. These lumenal cells probably also have some secretory activity. The cells of the outer row are larger, though the nucleus is smaller than those of the inner row. The cytoplasm is markedly vacuolated and the nucleus stains darkly. Nucleoli are rarely seen. These cells are similar in appearance to the myoepithelial cells of the normal duct. The material within the lumen is granular and shows well-marked eosinophilia in sections stained by a routine haematoxylin and eosin method, and it also stains very strongly with the periodic acid-Schiff technique.

The occurrence of cystic structures with the two rows of cells regularly disposed around the lumen in the manner just described is the exception rather than the rule, for in the great majority of cases one or other cell type preponderates. Thus in Fig. 3 the cystic structure towards the lower part of the field is lined by cells of the myoepithelial type whilst most of the other cysts are lined only by cells of the lumenal type. A point of note is the difference between the contents in these two types of cyst. In those lined by myoepithelial cells the contents are not markedly eosinophilic and are only weakly P.A.S.-positive.

Apart from arrangements of the types just mentioned, where the cysts are bounded by one or other of the cell types alone and apart also from those instances in which there is a regular arrangement of the cells in two layers, as in Fig. 2, various other configurations may also be encountered. Thus in Fig. 4 the large duct-like structure in the centre of the field is lined by a single layer of lumenal cells, while the myoepithelial cells next to this layer are present in sheets in the lower part of the field. On the other hand, above the duct, these cells have undergone apparently degenerative changes and only cell remnants in a mucoid matrix are to be seen. In some cases the myoepithelial cells predominate, with the presence of a good deal of mucoid intercellular material (Fig. 5).

As a further possibility there may be dilatation of the duct-like spaces rather than proliferation of cells. When this proceeds to an extreme extent, the lace-like pattern shown in Fig. 6 results. In other cases the cystic dilatation leads to the formation of structures which are much more duct-like in appearance (Fig. 7). Another expression of duct formation is that illustrated in Fig. 8. Here the tumour consists almost entirely of compressed ductular structures lined by a double row of cells disposed in a very regular manner.

Cyst-like spaces may also result from the enclavement of areas of stroma, or of mucoid material which is produced by the tumour cells in proximity to the stroma. When such areas, because of the plane of section, appear to be enclosed by epithelium the resemblance to an epithelial cyst is close. However, serial sections show that such enclaved areas are due simply to the particular manner in which stroma and epithelium are juxtaposed. Thus in Fig. 10 the area at "A"

becomes " encircled " in further sections, by epithelium, assuming an appearance very similar to that seen at " B ". Furthermore, the contents of cystic spaces of this type are only very weakly P.A.S.-positive, in contradistinction to the spaces lined by lumenal cells (Fig. 9).

These variations in histological pattern, with the differing modes of cyst formation, account for the apparent discrepancies in the staining reactions of the mucoid material in the cystic spaces which have been noted by a number of workers (Lemaitre, 1938; Kramer and Som, 1939; MacDonald, Moersch and Tinney, 1945; Belsey and Valentine, 1951). Recently, however, Azzopardi and Smith (1959) have shown that there are, in fact, histochemical differences between the mucins in the different types of spaces. In the duct-like structures the mucin is strongly P.A.S.-positive and is not metachromatic, whilst in what they term the pseudo-acini (i.e., the spaces bordered by myoepithelial cells) the mucin is only weakly P.A.S.-positive but is strongly gamma-metachromatic, showing marked reduction of the metachromasia after incubation with hyaluronidase. The hyaline material which appears to be formed in proximity to the stroma is P.A.S.-positive to a moderate degree and is rather less gamma-metachromatic. Methylene blue extinction confirms these differences.

Comparable histochemical observations have been made in the case of mixed tumours. Some authors, for example Hemplemann and Womack (1942), have taken this to indicate that mixed tumours are of diploblastic origin, while others (Grishman, 1952) consider the connective tissue type of mucin to be produced by myoepithelial cells. The work of Erichsen (1955) and Cotchin (1958) on mammary neoplasms in the bitch provides additional evidence that mucin produced by myoepithelial cells is of what is generally considered to be connective tissue type.

Solid patterns.—Solid types of growth are much less common than the cribriform pattern and generally a part only of the growth is arranged in this manner, the remainder being of the more usual architecture. However, occasionally an almost completely solid type of growth is encountered. Such growths may be difficult to identify, though thorough examination will generally reveal small areas here and there exhibiting more characteristic traits. Compression of ductular structures, as in Fig. 8, may lead to a practically solid type of growth, but more often the appearances are due to the tumour cells forming continuous sheets. Areas of necrosis tend to occur in solid growths (Fig. 11). Mitotic figures do not occur with any frequency in either solid or cystic tumours.

Two further uncommon variants may be mentioned. In one the cells are predominantly fusiform or spindle-shaped, the appearance in some fields being reminiscent of a neurinoma, with what appears to be palisading of the nuclei (Fig. 12 and 13). In the other the bulk of the tumour is composed of lumenal cells in an acinar arrangement. Only in a few areas at the edge of the tumour are myoepithelial cells to be seen grouped around the tubules and giving a clue to the cylindromatous nature of the growth (Fig. 14).

Stromal changes.—Stromal changes are sometimes prominent in cylindroma. A rather scanty fibrous stroma is the commonest finding (Fig. 1) but often the stroma is more abundant and frequently hyaline is present. The hyaline is either changed stromal connective tissue or it represents a product of the tumour cells, being laid down in proximity to the stroma. Hyalinization is often associated with a breaking-up of the cribriform pattern to form smaller cell groups

(Fig. 15). The deposition of mucinous material in proximity to the stroma, or the replacement of the stroma itself by mucoid, is also not uncommon. However, the tumour cell masses generally remain sharply defined and do not merge with the mucoid material, as occurs so frequently, and typically in mixed tumours. Thus in Fig. 16 the cell groups have quite distinct outlines, and are clearly demarcated from the stroma, which is almost entirely mucoid. Occasionally, however, there are encountered tumours in which the epithelial cells appear to "melt" into the mucoid in much the same way as occurs in mixed tumours. This is shown in Fig. 17, and much of the tumour from which this section was prepared showed similar changes.

Cyst formation in the stroma has already been discussed.

Differentiation between cylindroma and mixed tumour.—In the great majority of cases there is no difficulty in differentiating between typical examples of cylindroma and typical mixed tumours. Occasionally, however, instances occur where the distinction is not so readily made. One source of possible confusion is the mucoid change which is a typical feature of the mixed tumour and sometimes also occurs in the cylindroma. However, the distinguishing feature, as already pointed out, is the clear demarcation of epithelial cells from mucoid intercellular material in cylindroma, whereas in mixed tumour the epithelial cells appear to blend with the mucin. The occasional cylindroma in which mucoid intercellular change appears also to involve the epithelial cells, as shown in Fig. 17, may be confused with mixed tumour. A very unusual example of this type of mucoid change in a cylindroma is shown in Fig. 18. The tumour, from the parotid, showed on macroscopic examination a small nodule of different appearance to the rest of the growth, measuring just under 1 cm. in diameter and situated at one pole. On section the greater part of the tumour showed the typical cribriform pattern of cylindroma but the structure of the nodule, on preliminary examination, appeared very similar to that of a mixed tumour, consisting of scattered groups of epithelial cells and ductular structures in a completely mucoid matrix. Serial sections of the entire area showed the nodule to be clearly demarcated by a distinct fibrous capsule from the rest of the tumour, with which no connection could be demonstrated. However, more detailed examination showed the cells to be of typical cylindromatous type and clearly demarcated from the surrounding mucoid material.

Another type of configuration in cylindroma that shows some similarity to that seen in mixed tumour is the pattern illustrated in Fig. 5. This is comparable to those mixed tumours in which the cells tend to be of stellate shape. Foote and Frazell (1953) refer to such areas in mixed tumours as "pseudo-adenoid cystic", and found them in about 10 per cent of their cases, usually in limited amount. Duct formation in cylindroma (Fig. 7) may also produce appearances very similar to that seen in some mixed tumours.

In a small number of cases it may be impossible to decide whether a given tumour should be classified as mixed or as cylindroma.

Relationship to spheroidal cell carcinoma.—Ringertz (1938) and some other writers thought it possible to distinguish between benign and malignant cylindromas, those growths in which cyst formation was evident tending to belong to the former class and the solid tumours to the latter. Subsequent studies, however, have not shown any correlation between histological pattern and behaviour. The majority of tumours, whatever the histological picture, follow the same

type of course. This tends to be rather prolonged and is characterized by slow but persistent infiltration, growth along nerves often being a prominent feature. Metastases occur relatively late in the course of the disease and the secondary deposits generally show the same histological appearance as the primary growth. The same applies to local recurrences, even after a number of years. In some cases, however, local recurrences or metastases may show quite a different picture, presenting as spheroidal cell carcinoma from the histological point of view, and clinically assuming a more frankly carcinomatous course. Fig. 19 is an example of this type of growth. The primary growth, in the trachea, showed the typical cribriform pattern. The section illustration was from a local recurrence.

Histogenesis

Theories of origin of mucous gland tumours in general have been dealt with extensively in the literature, but there is very little with regard to cylindroma in particular.

An early view was that of Krompecher (1908), who considered the tumour to be akin to the basal cell carcinoma of the skin, both clinically and pathologically, and for this reason he used a corresponding nomenclature. Other authors have also referred to cylindroma as basal cell carcinoma or basalioma, either because such terms have appeared descriptively appropriate or because they have attributed a mucosal basal cell origin to the growth (Beck and Guttman, 1936). However, most authors who deal with the question of origin are agreed that the cylindroma arises directly from mucous, salivary or other glands, though there is some doubt as to which elements of the glands are responsible. This view is supported by the finding of tumour originating, or apparently originating, from gland elements, though reports of this are remarkably scanty, considering the number of tumours recorded in the literature. However, McDonald, Moersch and Tinney (1945) state that in one of their six cases the tumour appeared to be arising from mucous glands in the bronchial wall and Reid (1952) has also seen tumour originating in the duct of a bronchial gland. Russell (1955) suggests that the tumour arises or appears to arise from dilated mucous gland ducts.

In the neoplasms reported in this study, the close proximity of tumour to relatively normal mucous glands was noted in a number of cases. Mostly there was a clear demarcation between tumour and normal tissue, but sometimes growth and gland were intermingled. In such cases it might well have been the case that normal glandular tissue was being invaded by tumour, rather than giving rise to it, though in one lesion there appeared to be an actual transition between tumour tissue and normal gland.

The so-called "transitional lobules" have been thought to constitute transitional stages between normal glandular tissue and tumour. These lobules are aggregations of glandular tissue showing some dilatation of the ducts and degenerative changes in their lining cells and in the cells of adjacent alveoli. Chronic inflammatory infiltration is present in the stroma. These changes have been described in connection with mixed tumours though not so far as can be ascertained in cylindroma. Fig. 20 shows an example of this type of change in one of our cases, but we agree with Ringertz (1938) that the changes are degenerative and not neoplastic.

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REFERENCES

AHLBOM, H. E.—(1935) *Acta Radiol.*, Suppl. 23.
AZZOPARDI, J. G. AND SMITH, O. D.—(1959) *J. Path. Bact.*, **77**, 131.
BECK, J. C. AND GUTTMAN, M. R.—(1936) *Ann. Otol., etc., St. Louis*, **45**, 618.
BELSEY, R. H. R. AND VALENTINE, J. C.—(1951) *J. Path. Bact.*, **63**, 377.
BILLROTH, T.—(1859) *Virchows Arch.*, **17**, 357.
COTCHIN, E.—(1958) *J. comp. Path.*, **68**, 1.
DOCKERTY, M. B. AND MAYO, C. W.—(1942) *Surg. Gynec. Obstet.*, **74**, 1033.—(1943) *Surgery*, **13**, 416.
ENGELBRETH-HOLM, J.—(1944) *Acta chir. scand.*, **90**, 383.
ENTERLINE, H. T. AND SCHOENBERG, H. W.—(1954) *Cancer*, **7**, 663.
ERICHSEN, S.—(1955) *Acta. path. microbiol. scand.*, **36**, 490.
FOOTE, F. W., JR. AND FRAZELL, E. L.—(1953) *Cancer*, **6**, 1065.
FRAZELL, E. L.—(1954) *Ibid.*, **7**, 637.
GODTFREDSEN, E.—(1948) *Brit. J. Ophthal.*, **32**, 171.
GRISHMAN, E.—(1952) *Cancer*, **5**, 700.
HARRISON, K.—(1956) *Ann. R. Coll. Surg. Engl.*, **18**, 99.—(1957) *Ann. Otol., etc., St. Louis*, **66**, 459.
HEMPLEMANN, L. H., JR. AND WOMACK, N. A.—(1942) *Ann. Surg.*, **116**, 34.
KRAMER, R. AND SOM, M. L.—(1939) *Arch. Otolaryng., Chicago*, **29**, 356.
KROMPECHER, E.—(1908) *Beitr. path. Anat.*, **44**, 88.
LEMAITRE, Y.—(1938) *Ann. Oto-laryng.*, **57**, 185.
LENNOX, B.—(1960) in 'Recent Advances in Pathology', 7th Ed., edited by C. V. Harrison. London (J. & A. Churchill), p. 9.
LEVER, W. F.—(1948) *Arch. Derm. Syph., N.Y.*, **57**, 332.
MCDONALD, J. R. AND HAVENS, F. Z.—(1948) *Surg. Clin. N. Amer.*, **28**, 1087.
Idem, MOERSCH, H. J. AND TINNEY, W. S.—(1945) *J. thorac. Surg.*, **14**, 445.
MALASSEZ, M. L.—(1883) *Arch. Phys. norm. path.*, 3me série, **1**, 186.
MONTANUS, W. P.—(1938) *Surgery*, **4**, 423.
MULLIGAN, R. M.—(1943) *Arch. Path. (Lab. Med.)*, **35**, 357.
NEW, G. B. AND CHILDREY, J. H.—(1931) *Arch. Otolaryng., Chicago*, **14**, 669.
PATEY, D. H. AND THACKRAY, A. C.—(1958) *Brit. J. Surg.*, **45**, 477.
QUATTLEBAUM, F. W., DOCKERTY, M. B. AND MAYO, C. W.—(1946) *Surg. Gynec. Obstet.*, **82**, 342.
RANGER, D., THACKRAY, A. C. AND LUCAS, R. B.—(1956) *Brit. J. Cancer*, **10**, 1.
RAWSON, A. J., HOWARD, J. M., ROYSTER, H. P. AND HORN, R. C., JR.—(1950) *Cancer*, **3**, 445.
REID, J. D.—(1952) *Ibid.*, **5**, 685.
RIBBERT, M. W. H.—(1907) *Dtsch. med. Wschr.*, **33**, 126.
RINGERTZ, N.—(1938) *Acta Otolaryng., Stockh.*, Suppl. 27.
RUSSELL, H.—(1955) *Brit. J. Surg.*, **43**, 248.
SPIES, J. W.—(1930) *Arch. Surg., Chicago*, **21**, 365.
STEWART, F. W.—(1946) *Surgery*, **19**, 74.
Idem, FOOTE, F. W. AND BECKER, W. F.—(1945) *Ann. Surg.*, **122**, 820.
WATSON, W. L.—(1935) *Amer. J. Roentgenol.*, **34**, 53.

EXPLANATION OF PLATES

FIG. 1.—A commonly occurring pattern in cylindroma. The tumour consists of cell masses containing cystic spaces, giving rise to a cribriform appearance. ×60.

FIG. 2.—High power view of a cylindroma, showing the two cell types arranged as in a normal mucous gland duct. ×330.

FIG. 3.—To show the two types of cellular arrangement around cystic spaces. ×250.

FIG. 4.—A duct lined by lumenal cells at the centre of the field, with myoepithelial cells above and below. ×330.

FIG. 5.—The cells in this field are nearly all of myoepithelial type, with a considerable amount of intercellular mucoid material. ×95.

FIG. 6.—A typical appearance in cylindroma; well-marked cribriform pattern. ×45.

FIG. 7.—To show the formation of rather irregular duct-like spaces. ×95.

FIG. 8.—To show compressed duct-like structures. ×95.

FIG. 9.—Darkly stained contents of lumenal cysts contrasted with weakly P.A.S.—positive stromal cysts. ×185.

FIG. 10.—Mode of formation of stromal cysts. "A" indicates an area of stroma about to become enclaved by epithelium; "B" indicates a similar area, completely encircled. ×185.

FIG. 11.—Area of necrosis in a predominantly solid growth. ×60.

FIG. 12.—Bundles of elongated cells reminiscent of a neurinoma in the upper part of the field, with more typical cylindromatous pattern below. ×100.

FIG. 13.—High power view of the spindle cell area in Fig. 12. ×190.

FIG. 14.—Field at the edge of a tumour which for the most part was composed of cells of lumenal type. ×190.

FIG. 15.—Breaking-up of the cribriform pattern associated with hyalinisation. ×45.

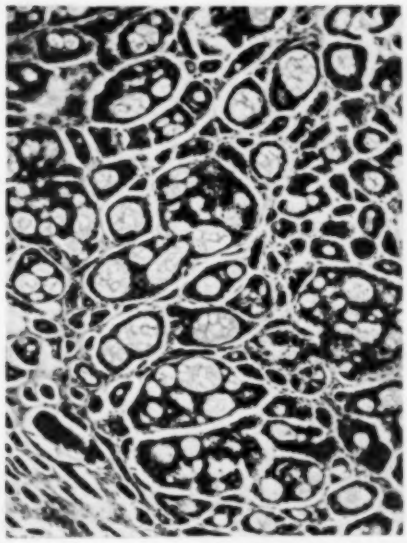
FIG. 16.—Sharply defined cell groups with intervening mucoid stroma. ×95.

FIG. 17.—Epithelial cells in a cylindroma appearing to melt into the mucoid. ×95.

FIG. 18.—Distinct nodule of growth in a parotid cylindroma. ×3.

FIG. 19.—Anaplasia in the local recurrence of a formerly well differentiated cylindroma. ×95.

FIG. 20.—A "transitional" lobule of mucous glands, adjacent to a cylindroma. ×95.



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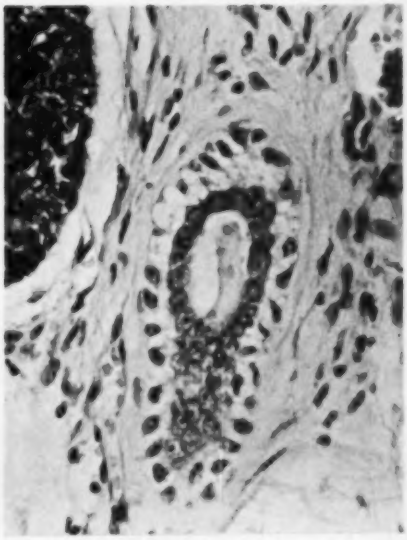

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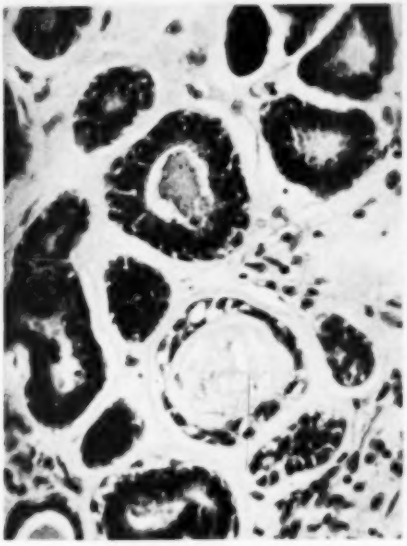

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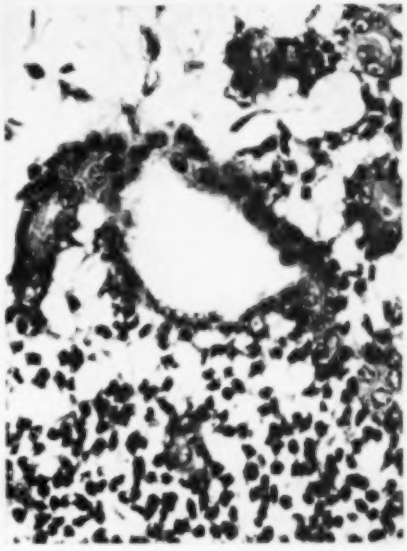

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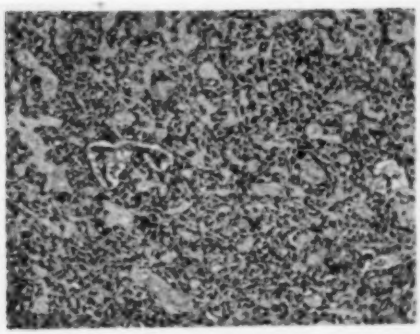

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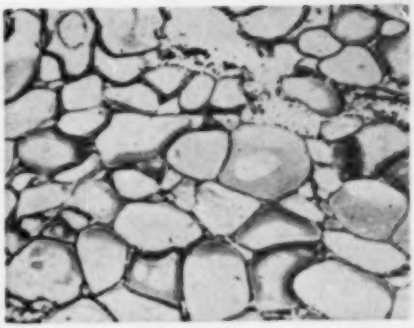

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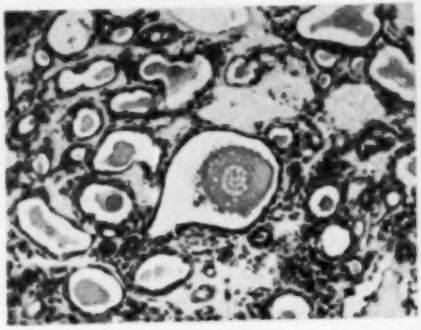

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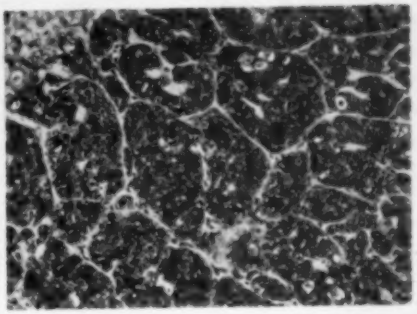

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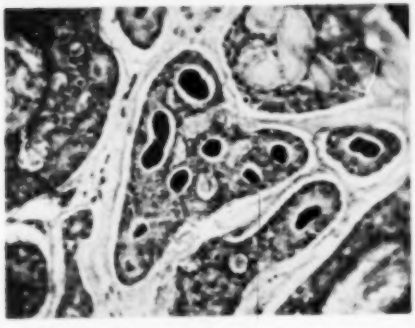

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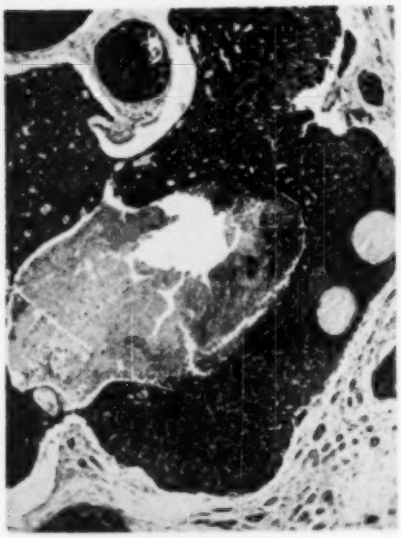

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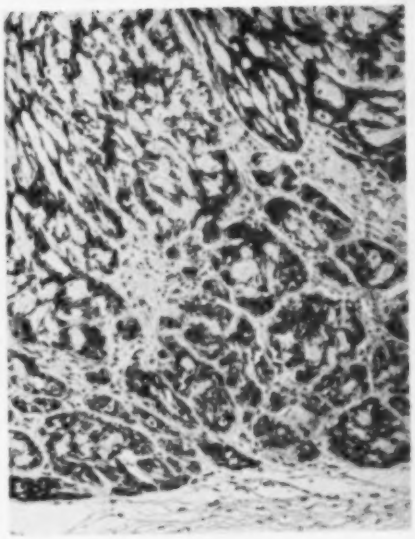

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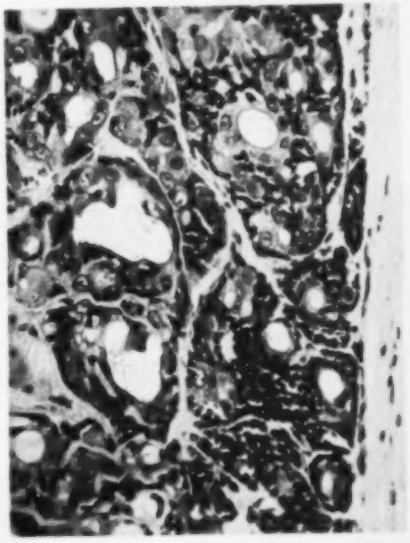

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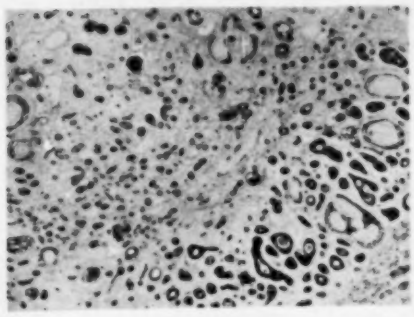

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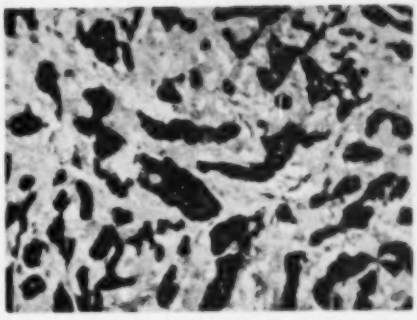

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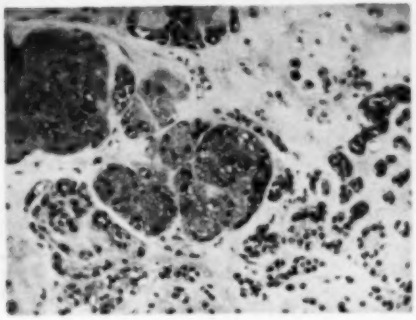

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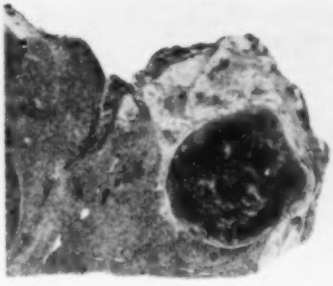

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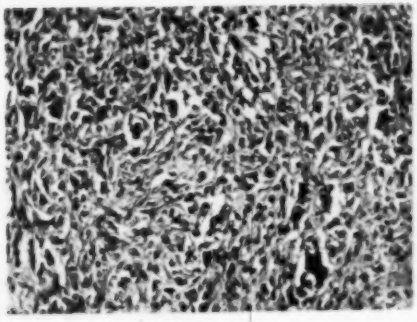

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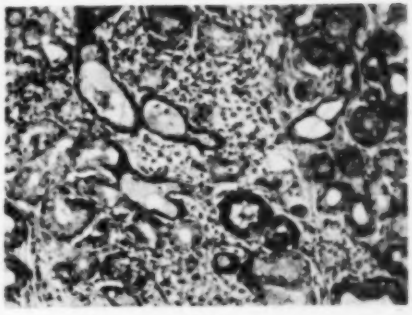

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LYMPHOSARCOMA IN THE OVARY IN YOUNG AFRICAN GIRLS IN NIGERIA

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LYMPHOSARCOMA is generally considered to be a disease predominantly of middle and old age though it is not rare in youth. The occurrence of lymphosarcoma in young African girls involving the ovaries as the most conspicuous site is of such frequency at University College Hospital, Ibadan, as to warrant description. During the years 1958 and 1959 the total number of malignant tumours among 1286 post-mortem examinations at this hospital was 107. The diagnosis of lymphosarcoma or reticulum cell sarcoma was made in 25 cases and in 7 of those the patients were girls under 14 years old.

TABLE I.—*Age and Sex of 25 Post-mortem Cases of Lymphosarcoma and Reticulum Cell Sarcoma*

Age in years	1–4	5–14	15–24	25–34	35–44	45–54	"Adult" Age not stated
Males	0	5	1	2	5	0	1
Females	0	7	0	2	1	1	0

Lymphosarcoma was the commonest of the 17 malignant tumours that were found at necropsy in children. Other tumours seen were 1 sarcoma of the urinary bladder, 1 medulloblastoma, 1 retinoblastoma, 1 Wilms tumour and 1 astrocytoma. In only one of the seven girls under 14 years with lymphosarcoma were the ovaries unaffected. No tumours of the testis were found in boys. The main clinical features and the necropsy findings in the six African girls who had ovarian tumours which proved to be lymphosarcomas were as follows :—

Case 1

S.S. (21649), aged 5 years, was admitted to hospital because of proptosis of the right eye with ophthalmoplegia and bilateral masses which were palpable in the lower abdomen. X-rays of the skull showed erosion of the posterior clinoid processes. The haemoglobin concentration was 8·2 g. per 100 ml. and the leucocyte count was 11,100 per cu.mm., the white cells being normal in type and distribution.

Necropsy.—The dura mater was under tension and the convolutions of the surface of the brain were flattened. There was a deposit of white tumour tissue in the pituitary fossa extending into the orbit on the right side and into the middle cranial fossa. The right and left ovaries were replaced by large masses of homogeneous white tumour tissue approximately 10 cm. in diameter and weighed 245 g. and 225 g. respectively. The uterus and Fallopian tubes were normal. Adjacent

to, but separate from the right ovarian mass there was a large diffuse retroperitoneal sarcoma extending from the pelvis towards the liver. The lymph glands of the thorax and abdomen were not enlarged. The stomach and intestines were normal. The spleen was enlarged but was of normal appearance. The liver, kidneys, ureters, bladder, pancreas and adrenal glands were normal. The heart and lungs were normal.

Case 2

M.A. (20059), aged 8 years, had been paraplegic for 8 days. X-rays showed a shadow at the level of the fifth thoracic vertebra suggestive of a para-vertebral abscess. The haemoglobin was 8 g. per 100 ml. and the white cell count 5400 per cu.mm. with a normal differential count. A costo-transversectomy was performed and at operation enlarged lymph glands were noted. The patient deteriorated steadily. Seven weeks after operation a pathological fracture of the right femur occurred and there was also a secondary tumour in the right tibia. She died the next day.

Necropsy.—There was diffuse infiltration of the bone and soft tissues of the upper thoracic spine by soft white neoplastic tissue. Mediastinal lymph glands were enlarged. The heart and lungs showed no abnormality. Both ovaries were completely replaced by soft white tumour tissue ; each measured 7 cm. in diameter. The rest of the genital organs were normal. Both kidneys were greatly enlarged and were almost entirely replaced by soft white tumour tissue (Fig. 1). The adrenal glands were replaced by tumour. All the para-aortic lymph glands were greatly enlarged. The liver and spleen were normal. The surface of the brain showed a slight flattening of the convolutions. The pituitary gland was replaced by a large deposit of tumour which extended into both cavernous sinuses.

Case 3

M.J. (22643), 6 years old, was admitted because of a swelling of the left upper jaw. The submandibular and cervical glands were swollen and the spleen was enlarged. X-rays showed a tumour in the left maxilla and an osteolytic soft tissue tumour in the central area of the mandible near the symphysis menti. Haemoglobin 10·7 g. per cent, leucocyte count 8000 per cu.mm., polymorphonuclears 69 per cent, lymphocytes 27 per cent, eosinophils 4 per cent.

Necropsy.—There was a nodule of white tumour 1·5 cm. in diameter in the anterior wall of the left ventricle. The lungs were normal. The mediastinal glands were not enlarged. The left kidney was replaced by a large homogeneous mass of tumour ; the right kidney contained several tumour nodules up to 2 cm. in diameter. The ovaries were 7 cm. and 8 cm. in diameter and consisted of homogeneous white tissue (Fig. 2, 5). The spleen contained several well defined tumour nodules. Lymph glands were enlarged in the splenic hilum and in the transverse mesocolon but not elsewhere. The skull and brain were normal.

Case 4

A.A. (25487) aged 8 years, had had oedema of the left leg for eight weeks and was admitted to hospital with a discharging sinus in the groin at the site of a biopsy. There was a mass in the right iliac fossa and a nodule was present on the bridge of the nose. X-rays showed diffuse involvement of the diaphysis of the left

tibia and fibula. Haemoglobin 11·5 g. per cent, leucocytes 5300 per cu.mm. She died on the day after admission.

Necropsy.—Apart from some congestion, the lungs and heart were normal. Both ovaries were enlarged to about 8 cm. in diameter with smooth glistening surface and were composed of homogeneous white tumour tissue (Fig. 3). Both kidneys contained many discrete white nodules up to 2 cm. in diameter (Fig. 5). The para-aortic lymph glands were slightly enlarged and of uniform white consistency and there were several enlarged glands in the left inguinal region. The mediastinal glands were normal. The liver was slightly fatty ; the spleen was normal. The nodule in the bridge of the nose consisted of a white tumour lying in the subcutaneous tissue and eroding the underlying bone.

Case 5

F. A. (36731), 9 years old, gave a history of abdominal swelling for two years and had developed pain in the abdomen recently. There were multi-lobulated masses arising from the pelvis and reaching the umbilicus. X-rays showed no tumours in the bones nor in the chest. Haemoglobin 7·8 g. The leucocyte count was not done. No abnormality was noted on a thin blood film. A laparotomy was performed and inoperable ovarian tumours were found.

Necropsy.—The heart and mediastinal lymph glands were normal. There was basal bronchopneumonia in the right lung. Two large solid masses in the pelvis replaced the ovaries. The right was 25 cm. and the left 10 cm. in diameter. The lymph glands alongside the abdominal aorta were enlarged. The kidneys were normal and no abnormality was present in the liver, spleen, adrenals or pancreas. The skull, brain and meninges were normal.

Case 6

A. Y. (39824), aged 11 years, was an ill, pale, wasted child who complained of a swelling on the left side of the abdomen which was said to have been first noticed 25 days before admission. On examination there was a large mass in the left upper abdomen and several other smaller masses elsewhere in the abdomen. X-rays of the chest and of the jaws were normal. An intravenous pyelogram showed distortion of the renal pelvis on both sides. Haemoglobin 7·4 g. per cent ; leucocyte count 11,500 per cu.mm. with polymorphonuclear leucocytosis.

Necropsy.—The thyroid gland was diffusely infiltrated by white tumour tissue ; the heart was normal and the lungs showed congestion. The mediastinal glands were not enlarged. The mass in the left hypochondrium consisted of the enlarged congested spleen together with a large diffuse mass of white tumour tissue lying in the omentum between the spleen and the stomach. There were several infarcts in the spleen. The para-aortic and mesenteric glands were greatly enlarged. Both kidneys were enlarged and the greater part of their substance was replaced by numerous nodules of white tumour. The ovaries were replaced by rounded tumour masses (Fig. 4). There was also a deposit in the anterior abdominal wall in the hypogastrium. The liver was cirrhotic. It was shrunken and nodular but contained no tumour. The skull, brain and meninges were normal.

In addition to the post-mortem cases described, we have seen during the past two and a half years the excised ovaries or ovarian biopsies of eight patients aged 8 to 16 years who had abdominal swellings and were found at laparotomy to have

TABLE II.—*Site of Tumour Deposits in Six Post-mortem Cases*

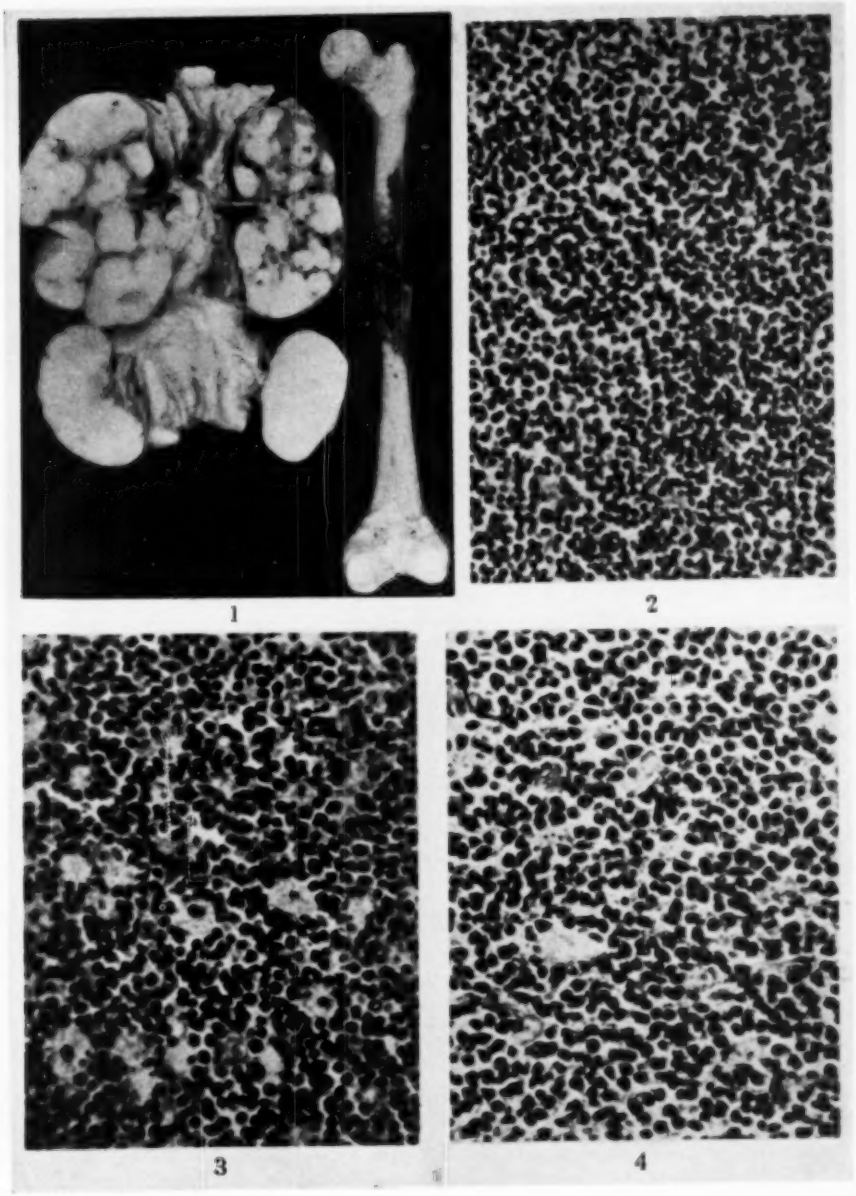
	Case number					
	1	2	3	4	5	6
Cranium	+	+	−	−	−	−
Jaws	−	−	+	−	−	−
Other Bones	−	+	−	+	−	−
Lymph Glands						
Abdominal	−	−	+	+	+	+
Peripheral	−	−	+	+	−	−
Kidneys	−	+	+	+	−	+
Adrenals	−	+	−	−	−	−
Spleen	−	−	+	−	−	−
Liver	−	−	−	−	−	−
Heart	−	−	+	−	−	−
Thyroid	−	−	−	−	−	+
Ovaries	+	+	+	+	+	+

ovarian tumours. In six cases there were tumour deposits in sites other than the ovary; one had paraplegia due to an extra-dural deposit in the spinal canal; in another case the intravenous pyelogram showed alteration of the pattern of the calyces suggesting deposits in the kidney. The blood count was done in all but one of the cases; there was no evidence of leukaemia. Three of the patients have not been seen again since they left hospital. One died two days after leaving hospital, one was moribund when seen six weeks after operation and two had multiple tumours when last seen. One patient had an ovarian tumour 10 cm. in diameter histologically identical to the other cases. A right ovariotomy was done and ten months later she was well with no evidence of tumour on clinical examination. Only two other ovarian tumours from young girls were examined histologically in the same period. Both were cystic teratomas.

Histologically the tumours were similar in every case. They were composed of sheets and masses of cells with small round hyperchromatic nuclei and scanty cystoplasm. The tumour cells lay in a reticulin network. Patches of necrosis were seen in some places. There was some slight variation in the size, shape and depth of staining of the nuclei and moderate numbers of mitoses were present. Scattered throughout the tissue there were larger reticulum cells with single nuclei and abundant pale-staining cytoplasm. In places because of shrinkage of the reticulum cell, or absence of its nucleus due to artefact, a false appearance of rosettes was formed. No neurofibrils could be detected. Normal ovarian tissue appeared to have been completely replaced, no remnant being seen in any of the sections. The tumours in the lymph glands, kidneys, and other sites presented an identical histological picture. The histological appearances were those of lymphosarcoma.

EXPLANATION OF PLATES

FIG. 1.—The enlarged ovaries, the kidneys and the femur of case 2. $\frac{1}{3}$ natural size.
FIG. 2.—Section of the ovary of case 3. H. and E. ×145.
FIG. 3.—Section of the ovary of case 4. H. and E. ×145.
FIG. 4.—Section of the ovary of case 6. H. and E. ×145.
FIG. 5.—Ovaries of case 3. Natural size.
FIG. 6.—Kidneys of case 4. Natural size.



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DISCUSSION

It is now well recognised that lymphosarcoma is common in tropical Africa. Elmes and Baldwin (1947) found that 5·3 per cent of 1000 tumours in Nigeria were lymphosarcomas ; Edington (1956) reported that lymphosarcoma and reticulum cell sarcoma together formed 5·7 per cent of 1193 malignant tumours obtained from autopsies and biopsies in the Gold Coast, and Camain (1954) found 9·6 per cent of cases of lymphosarcoma among 1884 malignant tumours in French West Africa. In Uganda, Davies, Wilson and Knowelden (1958) have reported that, among cases recorded by the Kampala Cancer Registry, tumours of the lymphatic system are about twice as common as would be expected by comparison with the rates obtained by the Danish Cancer Registry. On the other hand, Steiner (1954) found that in 35,293 autopsies in a 35 year period at Los Angeles County Hospital the frequency of the combined lymphatic malignant diseases was less in Negroids than in Caucasoids. The frequency with which lymphosarcoma occurs in young African children has been noted by O'Conor and Davies (1960) who state that during the seven years 1952–1958 the Kampala Cancer Registry recorded 125 malignant tumours occurring in children up to the age of 14 years among which there were 57 cases of lymphosarcoma. Further, Rosenberg, Diamond, Dargeon and Craver (1958) point out that when lymphosarcoma occurs in childhood, extra-nodal sites " appear to be primary " more frequently than in adults, although in their series of cases there were no negroes.

We are not aware of any report dealing with the frequent occurrence of lymphosarcoma in the ovary in childhood. The condition appears to be uncommon in Europe not being mentioned by Harnett (1952), Haines (1958) or Symmers (1958). In a review of the literature pertaining to ovarian lymphosarcoma Nelson, Dockerty, Pratt and ReMine (1958) found only 4 cases of " so called primary " and 24 cases of obviously secondary disease. They added 6 cases of their own in which they considered that the clinical features pointed to an ovarian participation in a more generalised lymphoblastomatous process. None of the cases described occurred in children. Burkitt (1958) reported from Kampala 38 cases of a tumour involving the jaws of African children which O'Conor and Davies (1960) now consider to be lymphosarcoma. There were deposits in the thyroid, testis, heart, stomach, salivary gland, cranium, femur, liver and kidneys, but ovarian tumours are not mentioned in any of the cases. Davies (1959) mentions 5 cases of ovarian tumour occurring among 179 childhood cancers but the histological diagnosis of the ovarian tumours is not given. Two undifferentiated round cell tumours of uncertain nature which occurred in the ovaries of girls aged 10 and 15 years were reported by Davies and Wilson (1954), and Edington (1956) mentions two girls aged 6 years who had bilateral ovarian tumours which were considered to be dysgerminomas. O'Conor and Davies (1960) do not mention the ovaries in an analysis of the incidence of involvement of different organs in 18 autopsied cases of lymphosarcoma in childhood. In a report of a Cancer Survey in the Transvaal (1953–55) Higginson and Oettlé (1960) did not find evidence of a high frequency of lymphomas such as has been reported from East Africa. They found that lymphomas formed 6·2 per cent of neoplasms in Bantu children (0–9 years) but did not mention the occurrence of this tumour in the ovaries of adults or children.

In Ibadan, Nigeria, all but two of the 16 ovarian tumours occurring in young girls that have been examined histologically in the past 2½ years have been lympho-

sarcomas; among the seven girls who died in hospital from lymphosarcoma there was only one case in which the ovaries were not affected. During the same period necropsies were done on 4 adult women who had died of lymphosarcoma and 107 ovarian tumours from adult women were sectioned. Among these no case of lymphosarcoma involving the ovary was found.

In the absence of a census and civil registration of death we are not able to make any exact estimate of the frequency of ovarian lymphosarcoma in the population but we believe that the frequency of the disease in the post-mortem and biopsy material of this hospital suggests that it is more common in young African girls in Nigeria than has been recognised hitherto and that this may well be true of other parts of tropical Africa.

SUMMARY

Involvement of the ovaries is a common and conspicuous feature when lymphosarcoma occurs in young African girls in Ibadan, Nigeria. The majority of ovarian tumours in young girls here are lymphosarcomas. The same is not true of adult women.

We are grateful to the medical staff of the Department of Obstetrics and Gynaecology and to other surgeons and physicians for permission to use their clinical records, to Dr. W. P. Cockshott for radiological reports and to Mr. F. E. Speed of the Medical Illustration Unit, University College Hospital, Ibadan, for the photographs.

REFERENCES

BURKITT, D.—(1958) *Brit. J. Surg.*, **197**, 218.
CAMAIN, R.—(1954) *Bull. Soc. Pat. exot.*, **47**, 614.
DAVIES, J. N. P.—(1959) in 'Modern Trends in Pathology', ed. D. H. Collins, London (Butterworth & Co.), p. 153.
Idem AND WILSON, BARBARA A.—(1954) *E. Afr. med. J.*, **31**, 395.
Idem, WILSON, B. A. AND KNOWELDEN, J.—(1958) *Brit. med. J.*, **2**, 439.
EDINGTON, G. M.—(1956) *Brit. J. Cancer*, **10**, 595.
ELMES, B. G. T., AND BALDWIN, R. B. T.—(1947) *Ann. trop. Med. Parasit.*, **41**, 321.
HAINES, M.—(1958) in 'Cancer', ed. R. W. Raven, London (Butterworth & Co.), Vol. 2, p. 298.
HARNETT, W. L.—(1952) 'A Survey of Cancer in London'. London (British Empire Cancer Campaign).
HIGGINSON, J. AND OETTLÉ, A. G.—(1960) *J. nat. Cancer. Inst.*, **24**, 589.
NELSON, G. A., DOCKERTY, M. B., PRATT, J. H. AND REMINE, W. H.—(1958) *Amer. J. Obstet., Gynec.*
O'CONOR, G. T. AND DAVIES, J. N. P.—(1960) *J. Pediat.*, **56**, 526.
ROSENBERG, S. A., DIAMOND, H. D., DARGEON, H. W. AND CRAVER, L. F.—(1958) *New Engl. J. Med.*, **295**, 505.
STEINER, P. E.—(1954) 'Cancer: Race and Geography'. Baltimore (Williams & Wilkins Co.).
SYMMERS, W. ST C.—(1958) in 'Cancer', ed. R. W. Raven. London (Butterworth & Co.), Vol. 2, p. 448.

RESULTS IN THE TREATMENT OF CANCER OF THE BREAST BY INTERSTITIAL IRRADIATION OF THE PITUITARY

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In patients with advanced cancer, it is often not only difficult to prolong life, but also to be certain whether treatment has done this. The relief of symptoms, on the other hand, is easier to assess and in many cases not so difficult to accomplish.

Huggins and Scott (1945) described the effects of adrenalectomy in breast cancer. This operation apparently prolonged life and often relieved suffering. Soon, however, it became evident that this only occurred in a comparatively small number of patients. The proportion of remissions obtained in the accounts of published cases was never more than 40 per cent. The fact that so many patients were unaffected by adrenalectomy was explained, however, by the suggestions that the tumour was either not " hormone dependent ", or had become autonomous. Alternatively, there may have been incomplete removal of the sources of oestrogen production which may be either in accessory adrenal or ovarian tissue. In all the patients who benefited from the operation, reactivation of the disease occurred, in the majority of cases, within two years, though, exceptionally, a few remained well for periods as long as three or four years. The simplest explanations for this reactivation were that there had been a continuation of oestrogen secretion, or that the tumour was now being stimulated by pituitary hormones, or possibly, that it had become completely autonomous.

When, therefore, Luft and Olivecrona (1952) described the results of treatment of cancer of the breast by hypophysectomy, it seemed possible that this treatment might give better results than adrenalectomy. The more complete removal of oestrogenic hormones, as well as prolactin and somatotrophin, should, theoretically, raise the remission rate very considerably. It was necessary to accept the fact that adrenalectomy and hypophysectomy should only be undertaken in advanced cases for palliation and not used in the early cases as a curative procedure. The aim of treatment, in the advanced case, was to provide amelioration of the disease, with relief of symptoms, for two or even three years. It was decided, therefore, that only advanced cases, in whom all recognized palliative treatment had already been carried out without success, should be treated by pituitary ablation. These patients were specially selected for the trial of the new method of treatment and selection was based, entirely, upon the fact that all conventional methods had previously been tried.

Because the operation of hypophysectomy is a difficult procedure and complete hypophysectomy is difficult to obtain, it seemed reasonable to attempt to destroy the pituitary by interstitial irradiation.

History

The history of irradiation of the pituitary goes back thirty years or so. Henderson (1938) in reporting on 338 cases of pituitary adenomata treated by Cushing, described the implantation of radium, by the transphenoidal route, into patients with basophil adenoma. In 1936, Lodge described an approach via the orbit and ethmoid sinus to the sella turcica, in cases where the latter had become grossly expanded by a tumour ; after removal of as much of the growth as possible, radon seeds were implanted (Northfield, 1949). This same route is used by Bauer (1956) to insert a cannula into the gland.

External irradiation with conventional X-rays produces little or no effect upon this extremely radioresistant structure (Kelly *et al.*, 1951), a high energy proton beam can destroy the gland (Tobias *et al.*, 1958).

Other methods such as electrocoagulation and the injection of chemical or radioactive colloidal solutions, have also been tried. Electrocoagulation (Bauer, 1956) has not proved satisfactory because the electrode becomes covered with an insulating layer of charred tissue and further destruction is thus prevented. The injection into the gland of solutions of corrosive material, or of radioactive materials is dangerous because of the impossibility of limiting the fluid to the confines of the sella.

Radon seeds were the obvious choice, but experience has shown that their penetrating gamma rays cause serious damage to the optic nerve which may lead to total blindness in some cases (Forrest *et al.*, 1956 ; Westminster Hospital Report, 1956). It seemed reasonable, therefore, to try the effect of radioactive gold grains (gold–198) and these were first inserted in February 1955. This comparatively simple procedure from a technical point of view should be suitable for use in a seriously ill patient. Since the expectation of life following implantation would be unlikely to exceed three years, it would appear justifiable to accept the possible occurrence of delayed effects of irradiation following damage to the hypothalamus.

Rasmussen, Harper and Kennedy (1953) suggested from experimental evidence that yttrium 90, a pure beta ray emitter, would be a suitable source for producing localized destruction of the pituitary without damage to the surrounding structure. Their experimental findings were confirmed by Yuhl *et al.* (1955) who introduced yttrium pellets at craniotomy. Yttrium was therefore used for this purpose at the Royal Marsden Hospital in July 1956.

Between February 1955 and March 1958, 100 patients with metastatic carcinoma of the breast were treated by implantation of the pituitary. Gold–198 was used in 54 cases and yttrium–90 in 36. The remaining 10 patients had first one, and later, the other isotope implanted. Screened gold grains 2·5 mm. long, 0·8 mm. diameter and with a sheath of platinum 0·15 mm. thick were used at first, but later, unscreened rods 5·0 mm. long, and 0·8 mm. diameter were implanted.

Gold–198 emits mainly gamma rays which are less penetrating than those from radon and a small quantity of beta rays which do not penetrate, for the

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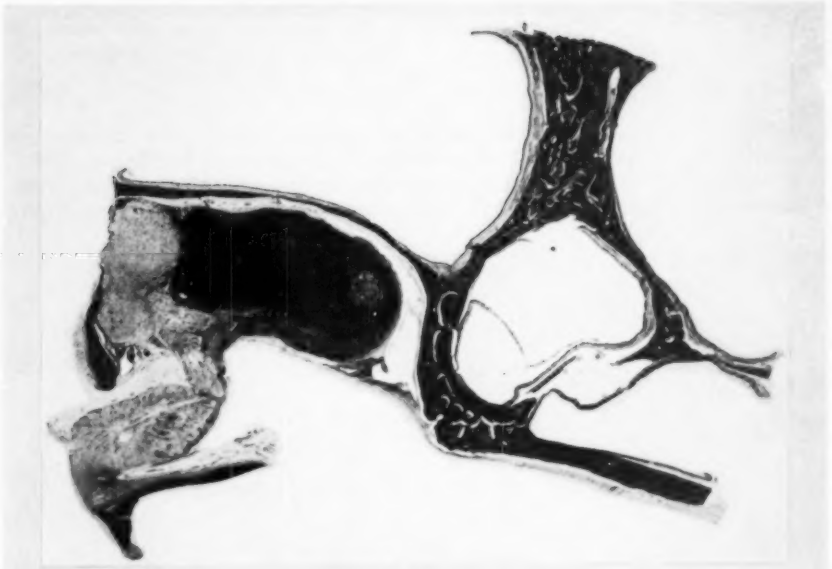
EXPLANATION OF PLATE

FIG. 3.—The pituitary occupies about three quarters of the depth of the fossa in this case.
FIG. 4.—Scaphoid anterior pituitary lying on the floor of the fossa.



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half life of the two isotopes is almost the same. Isodose curves (Fig. 1) show the much more rapid fall off with distance of the radiation from yttrium compared with that from gold.

Method

The operation was always carried out under bacteriostatic and antibiotic cover. This usually took the form of sulphadiazine, half a gramme six-hourly, which was started on the day before operation and continued for a further five days. In the earlier cases the nose was treated with streptomycin nasal drops,

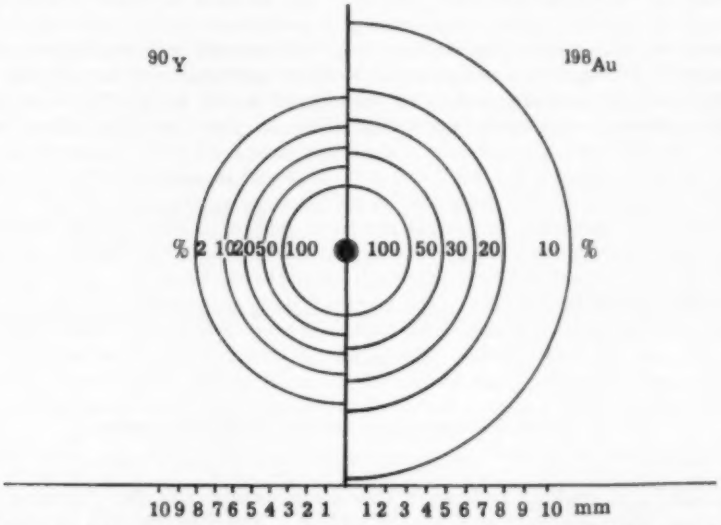

FIG. 1.—Isodose curves for ^{90}Y and ^{198}Au. (Doses equated at 3 mm. from source.)

for two or three days before operation, but this was discontinued later in the series. Cortisone 50 mg. daily was started on the day of operation ; in the earlier cases it was witheld until signs of adrenal insufficiency appeared but this was considered to be an unnecessarily severe test on the patient, and was abandoned. Antero-posterior and lateral radiographs of the skull were taken before operation. The anaesthetic was administered by a cuffed endotracheal tube passed through the mouth. The nose was packed before operation with gauze soaked in cocaine and adrenalin. The rods of either ^{198}Au or ^{90}Y were inserted through a cannula introduced into each nostril in turn. The position of the cannula was controlled by visualization on an image intensifier, working in two planes.

Postoperative

The patient was nursed in a horizontal position for 12 hours and then allowed to sit up. Cortisone was continued indefinitely and thyroid extract was given when signs of thyroid deficiency appeared two to three months after operation.

Complications

All patients developed headache but this was not usually severe and was easily controlled by simple analgesics. In a few cases, persistent diabetes insipidus made it necessary to give either pitressin tannate in oil or pitressin snuff. Bleeding was a common occurrence but was not often severe, though two cases required blood transfusion. Excessive bleeding was usually confined to those patients in whom the base of the skull was grossly involved by metastatic tumour. Optic atrophy occurred in 3 patients and must be assumed to be due to the implant, but unfortunately this could not be confirmed as post-mortem examinations were not carried out on these particular patients. In one case, however, blindness was discovered, at autopsy, to have been due to a metastasis in the optic nerve. The most common, disastrous complication was rhinorrhoea followed by meningitis which caused 10 deaths. Cerebrospinal fluid rhinorrhoea and meningitis, either separately, or together, occurred in 21 patients of which 9 had ^{198}Au and 12 had ^{90}Y. Six patients developed meningitis without previous rhinorrhoea and 5

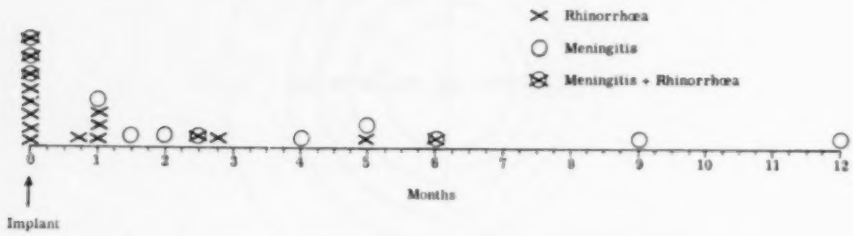

FIG. 2.—Onset of rhinorrhoea and meningitis following implant.

of these died. A striking feature of this complication was the variation in the lapse of time after implant before its occurrence. The diagram (Fig. 2) illustrating this includes two patients suffering from a disease other than cancer of the breast.

Antibiotic cover, as long as the leakage persisted, was the only treatment employed. Meningitis was extremely resistant to treatment and the clinical course varied from acute fulminating to chronic. At post-mortem examination, multiple adhesions with much fibrous exudate was commonly found and it was not surprising, therefore, that antibiotics had little effect in these cases. There are several possible causes of the cerebrospinal fluid leak. There was no correlation between the activity of the rods and the development of the rhinorrhoea, but there is no doubt that it occurs if rods are placed high up anteriorly. In this position they lie just under the diaphragma sellae and may cause it to necrose (Forrest, 1959, personal communication). Rhinorrhoea can, however, occur with properly placed rods and Forrest suggests that it is then due to overdosage, but this does not explain the immediate leak of cerebrospinal fluid which we have noticed on several occasions on the introduction of the cannula low down near the floor of the fossa. The cause, therefore, probably lies in abnormal anatomy and there are two variations from the normal which may be important. Firstly, the depth of the fossa, as shown radiologically, does not necessarily indicate the size of the gland which it contains (Fig. 3). The pituitary may

sometimes consist of no more than a flattened disc (Fig. 4) lying on the floor, and in such a case, the diaphragma is not stretched across the top but dips down to be closely applied to the upper surface of the gland. It is easy to see, therefore, that a rod placed in the middle of the fossa and judged to be in the middle of the gland, might in fact be lying above the diaphragma in the subarachnoid space. Secondly, the diaphragma sellae is often deficient. Mahmoud (1958) found this to be so in forty of one hundred fossae examined at autopsy. Sometimes the deficiency is quite large and it is in such cases that the subarachnoid space may extend down into the fossa.

Results

The assessment of the results of hypophysectomy is difficult. In many cases, the disease is apparently arrested—no new lesions appearing, but without any signs of healing of the original ones. In some patients marked subjective improvement occurred with no evidence of any objective remission. It was decided, therefore, only to assess those cases who have shown definite objective remissions but this does not include "arrest" of the disease. Many patients die from their disease within a short time of operation and these cannot, therefore, be assessed but in order to give an accurate picture of the series of 100 cases they have been included, and no case has been removed from the series on the grounds that it has been impossible to evaluate the patient, either because they have been lost to follow-up, or, because they died too soon. All complications are included and any death in which it is doubtful whether it was the result of the operation, has, nevertheless, been included in the series. In addition to this, no patient was refused treatment, either because they were unfit for operation, or because it was considered that the disease was too advanced. The results, therefore, must be considered to be as complete as possible.

Only two patients are alive, although twelve (11—^{198}Au, 1—^{90}Y) had objective evidence of regression and one of these survives 44 months after implant. The remainder lived from periods ranging from 7 to 40 months, with an average survival of 19·4 months. The survival of the 88 patients who did not respond is shown (Fig. 5); 65 were dead in six months and 13 lived for less than one month. The few who lived for relatively long periods remind one how chronic this disease can be.

In 5 cases only was pituitary implantation performed as the first planned treatment. Thirty-seven of the patients had had previous endocrine surgery, either oophorectomy alone, or combined with adrenalectomy. None of them showed improvement after pituitary implantation no matter what their response to the previous operation had been. This is not surprising in those cases treated by adrenalectomy, as response to pituitary ablation is unusual after this operation. There was only one case that had relapsed after successful oophorectomy, and pituitary implantation failed to induce further regression. It is obvious that no reasonable comparison can be made of the results in this series with those obtained by other methods of treatment.

Investigations

Apart from routine blood examinations and radiological surveys of the skeleton, the following investigations were carried out on these patients:

1. Urinary godadotrophin excretion.
2. Urinary oestrogen excretion.
3. Serum 17-ketosteroids.
4. Radioactive iodine uptake.

The urinary gonadotrophin and oestrogen assays were undertaken primarily in order to see whether there was any correlation between the preoperative levels and the results of treatment. It was also hoped to show whether there had been complete pituitary destruction by comparing the postoperative findings with those carried out before operation.

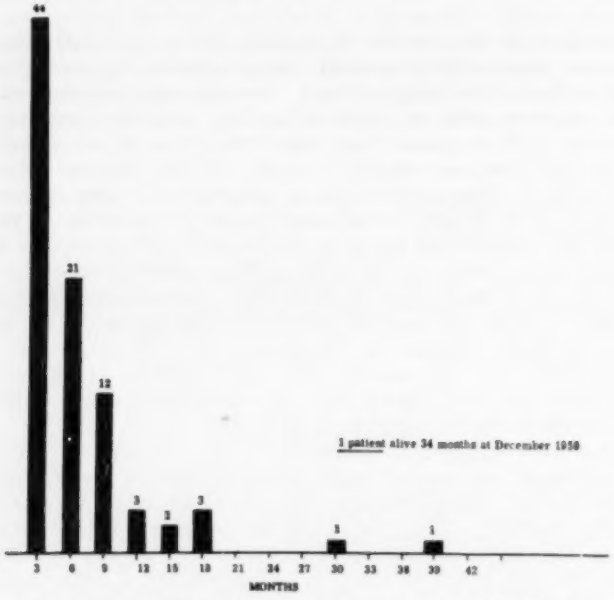

FIG. 5.—Survival after implant (non-responders) = 88 patients.

It is not the purpose of this paper to analyse in detail the results of these investigations, but some important points are noted. The estimation of gonadotrophin excretion in the urine is the only satisfactory test available at present for the measurement of pituitary function. The levels in premenopausal women tend to be low. They rise after the menopause and the highest figures are usually seen following castration. In any patient, however, whether premenopausal or postmenopausal, there are wide variations from day to day and some solitary tests are probably, therefore, valueless (Fig. 6). A fall to zero from a high value before pituitary destruction indicates a considerable diminution of pituitary function, but apart from a suggestion in those patients with moderately high levels of gonadotrophin excretion before operation that a good result is probable, we have not obtained any help from this estimation. The levels of oestrogen in

the urine are subject to considerable daily variation and furthermore, the values are influenced by the fact that many patients are having cortisone. We do not find that preoperative or postoperative oestrogen assays are of value in the choice of treatment.

The plasma 17-ketosteroid level usually falls after implantation but levels obtained in all outpatients were so low that this did not seem to be of any significance.

Radioactive iodine uptake studies have shown evidence of diminished thyroid function and have occasionally been of value in assessing depressed pituitary function in those patients whose preoperative gonadotrophin levels were low and in whom, therefore, no fall could be recorded after implantation.

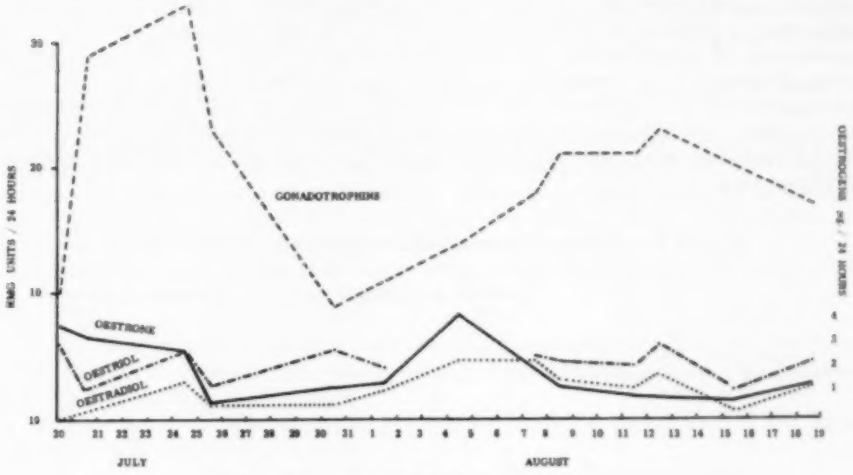

FIG. 6.—Post-menopausal woman, aged 53.

Pathology

The extent of destruction of the pituitary was estimated in 39 specimens obtained at post-mortem and examined histologically. In 4 the gland was totally destroyed—3 by ^{90}Y (11·9, 12·8, 8·6 mc) and 1 by ^{198}Au (80 mc). In 7 cases, the extent of destruction was estimated to be between 90 and 95 per cent, and the activity of the rods ranged from 34 to 115 mc of ^{198}Au and from 5·4 to 11·7 mc of ^{90}Y. The smallest amount of ^{90}Y known to have destroyed the pituitary was 8·6 mc and the largest amount (11·7 mc) had not destroyed all the gland. Thus, about 10 mc of ^{90}Y should be sufficient to produce total necrosis in most cases. In common with other workers, we have found that ^{198}Au is not efficient in producing total necrosis and in one case, 125 mc still left between 10 and 20 per cent of apparently viable cells.

We were able, in a few cases, to correlate the clinical response with the extent of necrosis found at autopsy and the pre- and post-implantation urinary gonadotrophin levels. In 2 of the 12 cases showing objective regression the destruction was 100 per cent in one and 95 per cent in the other and the gonadotrophin

levels fell to zero in both cases. Among the patients who did not respond there were 6 in which the extent of destruction was between 80 and 100 per cent and in all these the gonadotrophin excretion fell to very low levels after implantation. In the other 2 cases the amount of pituitary destroyed was small, 10 per cent in one and 33 per cent in the other, yet, there was considerable fall in the gonadotrophin excretion. One must, therefore, accept with caution a fall in urinary gonadotrophin excretion as evidence of complete histological destruction of the pituitary, although it probably reflects loss of function.

Discussion

Since there is considerable difference in the published figures given, both for surgical hypophysectomy and irradiation hypophysectomy, the result in comparable series of patients from the point of view of the remission rate, mortality and percentage of complications etc. are summarized in Table I. The number of cases of breast cancer treated by surgical removal of the hypophysis is 342. The average mortality is 7 per cent, the complication rate 15 per cent and the remissions 46 per cent. In 675 cases treated by interstitial irradiation of the pituitary, the mortality is 3 per cent, the complication rate 32 per cent and the average remission is 29 per cent.

TABLE I.—*Effects of Interstitial Irradiation in Breast Cancer*

Authors	Number of cases	Mortality (%)	Complications (%)	Beneficial response (%)
Fraser *et al.*, 1959	61	2	49	54
Ironside *et al.*, 1959	43	—	77	26
Greening *et al.* (this paper)	100	11	24	12
Forrest *et al.*, 1959	71	3	6	14
Bauer, 1956	400	1 (less than)	4	40 (approx.)
Total	675	3	32	29
	Surgical removal			
Ray and Pearson, 1959	109	3	12	50 (arrest 9%)
Kennedy *et al.*, 1956	34	9	32	53
Luft *et al.*, 1958	59	12	—	26 (arrest 14%)
Radley-Smith, 1960	70	10	30	42 (arrest 10%)
Atkins *et al.*, 1960	70	3	10	60
Total	342	7	15	46

Cerebrospinal fluid leakage is the main problem to be solved in order to make interstitial implantation of the pituitary safe. Since the leakage usually occurs when rods are placed high up in the fossa and rarely when they lie on the floor, high placement of the rods must be avoided and in order to do so the fossa should be approached horizontally. A horizontal approach via the nose is impossible in many cases and septal deviation and enlarged turbinates add to the difficulties of the operation. We have therefore changed over to the transethmoidal route used by Bauer (1956), and a trial of this method is being undertaken.

We also consider that the advantages of ^{90}Y over ^{198}Au are theoretical rather than practical. ^{90}Y with a short range and rapid fall off, requires to be placed very accurately and since the rods are now implanted low in the fossa, in all cases, it is preferable to use ^{198}Au since it has a somewhat greater range and will deliver an adequate dose to all parts of the gland. The amount of radiation required is not known with any degree of certainty but for purpose of a proposed trial we are using 50 mc of ^{198}Au.

Summary

Between February 1955 and March 1958, interstitial irradiation of the pituitary was carried out in 100 cases, using either ^{198}Au or ^{90}Y. When all treated patients are included in the series and the strictest possible criteria used the remission rate was 12 per cent, the mortality 11 per cent and the complication rate 24 per cent. The patients treated were those for whom no other method of treatment was available.

The operation itself is technically simple and in the majority of cases, no more of an ordeal than ovariectomy.

It is suggested that this method of treatment is, in certain cases, well worth continuing and that its usefulness could be increased considerably if the complication of rhinorrhoea were eliminated. Although it was originally undertaken only in advanced cases of breast cancer, it has other spheres of usefulness and may be helpful both in acromegaly and pituitary tumours.

A further trial of the method is being undertaken at present, using ^{198}Au grains implanted via the transethmoidal route.

We wish to thank all those authors who made their results available to us. Our thanks are also due to the Departments of Medical Art and Photography of the Royal Marsden Hospital.

Fig. 1, 2 and 3 (Ramsey, 1960) are reproduced by permission of the Royal Society of Medicine.

REFERENCES

Atkins, H. J. B., Falconer, M. A., Hayward, J. L., MacLean, K. S., Schurr, P. H. and Armitage, P.—(1960) *Lancet*, i, 1148.

Bauer, K. H.—(1956) *Arch. klin. Chir.*, **284**, 438.

Forrest, A. P. M., Blair, D. W., Peebles Brown, D. A., Stewart, Helen J., Sandison, A. T. Harrington, R. W., Valentine, J. M. and Carter, P. T.—(1959) *Brit. J. Surg.*, **47**, 61.

Idem, Peebles Brown, D. A., Morris, S. R. and Illingworth, C. F. W.—(1956) *Lancet*, i, 399.

Fraser, R. Joplin, G. F., Laws, J. W., Morrison, R. and Steiner, R. E.—(1959) *Ibid.*, i, 382.

Henderson, W. R.—(1938) *Brit. J. Surg.*, **26**, 811.

Huggins, C. and Scott, W. W.—(1945) *Ann. Surg.*, **122**, 1031.

Ironside, W. M. S., Moseley, K. D. and De Vos, W. N.—(1959) *Acta Un. int. Cancr.*, **15**, 1102.

Kelly, K. H., Felsted, E. T., Brown, R. F., Ortega, P., Bierman, H. R., Low-Beer, B. V. A. and Shimkin, M. B.—(1951) *J. nat. Cancer Inst.*, **11**, 967.

Kennedy, B. J., French, L. A. and Peyton, W. T.—(1956) *New Engl. J. Med.*, **255**, 1165.

LODGE, W. C.—(1936) *Brit. Med. J.*, ii, 1257.
LUFT, R. AND OLIVECRONA, H.—(1952) *Nord. med.*, **57**, 351.
Iidem, IKKOS, D., NILSSON, L. B. AND MOSSBERG, H.—(1958) 'Endocrine Aspects of Breast Cancer', edited by A. R. Currie. Edinburgh (Livingstone), p. 27.
MAHMOUD, M. E. S.—(1958) *Brit. J. Radiol.*, Suppl. 8.
NORTHFIELD, D. W. C.—(1949) *Proc. R. Soc. Med.*, **42**, 845.
RADLEY-SMITH, E. J.—(January, 1960) Personal communication.
RAMSAY, G. S.—(1960) *Proc. R. Soc. Med.*, **53**, 641.
RASMUSSEN, T., HARPER, P. V. AND KENNEDY, T.—(1953) *Surg. Forum*, **4**, 681.
RAY, B. S. AND PEARSON, O. H.—(1959) *Cancer*, **12**, 1.
TOBIAS, C. A., LAWRENCE, J. H., BORN, J. L., MCKOMBS, R. K., ROBERTS, J. E., ANGER, H. O., LOW-BEER, B. V. A. AND HUGGINS, C. B.—(1958) *Cancer Res.*, **18**, 121.
WESTMINSTER HOSPITAL REPORT.—(1956) *Rep. Brit. Emp. Cancer Campgn*, **34**, 173.
YUHL, E. T., HARPER, P. V., RASMUSSEN, T. B. AND BERGENSTAL, D. M.—(1955) *Surg. Forum*, **6**, 489.

THE EFFECTS OF A SENECIO ALKALOID (MONOCROTALINE) ON HUMAN EMBRYO LIVER IN TISSUE CULTURE

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THIS communication describes the effects of monocrotaline, a pyrrolizidine alkaloid, on human liver cells in tissue culture.

Seneciosis is a liver disease which is due to the ingestion of certain plants, mostly of the genus Senecio, containing alkaloids of the pyrrolizidine group and this condition has been a subject for research in this department for some time. Many workers, including Schoental and Head (1955), Bras and Hill (1956), Berry and Bras (1957), Schoental and Magee (1959) and Hill (1959), have described the various lesions produced both naturally and experimentally, but the pathogenesis of this disease is still obscure. Associated with other phenomena, several authors have observed enlarged parenchymal cells with single hypertrophied nuclei in the livers of affected animals (Harris, Anderson and Chen, 1942; Hill, 1959; Stephenson, unpublished). Bull (1955) has termed this condition "megalocytosis".

Neoplastic changes have been reported in rat livers after injection with pyrrolizidine alkaloids by Cook, Duffy and Schoental (1950) and Schoental, Head and Peacock (1954) and in fowls by Campbell (1956). An investigation at the cellular level, uncomplicated by vascular or other effects, was therefore thought to be worth investigating.

MATERIALS AND METHODS

A strain of human embryo liver cells (HuLi)* originally established by Westwood, McPherson and Titmuss in 1957 was used for most of these studies. One series of experiments was also performed with a strain of HeLa cells maintained for some time in this laboratory.

All the cells were cultured in a medium which contained the following ingredients:

Calf serum (deactivated at 60° C. for 30 min.)	15·0%
Lactalbumen hydrolysate	0·5%
Yeast extract	0·5%
Tryptic meat broth	5·0%
Peptic digest of sheep's blood	0·1%

Earle's saline to 100.

Antibiotics were added in concentrations of:

penicillin, 100 units/ml.
mycostatin, 200 units/ml.
neomycin, 0·2 mg/ml.
streptomycin, 20 units/ml.

* Obtained through the kindness of Dr. T. H. Flewett, Regional Virus Laboratory, Little Bromwich General Hospital, Birmingham, 9.

The medium was made up weekly from stock solutions which were discarded after six months and the freshly dissolved antibiotics were added just before use.

Stock cultures were grown on glass in pyrex feeding bottles incubated at 37° C. and re-fed weekly. Cells were removed from the glass by incubating for 5 min. with 0·1 per cent trypsin (Difco 1 : 250) in phosphate-buffered saline at pH 7·2. The cell suspension thus obtained was spun at 1000 r.p.m. for 5 min. After the supernatant liquid was removed, the cells were resuspended in fresh medium by stirring magnetically for 5 to 10 min., then dispensed into fresh bottles. Stock bottles received aliquots of 10 ml. with cells in the concentration of 2×10^5 cells/ml.

The experimental vessels were Sanders PM/3 vials with silicone rubber-lined screw caps, each containing a coverslip on which the cells settled. Two millilitres of the cell suspension together with the substance under test was seeded into each vial and incubated at 37° C. Tests were made with monocrotaline HCl, sterilized by Seitz filtration, in concentrations of 1, 2·5, 25, 50, 125, 250 and 500 μg./ml. Other hepatotoxic agents, used in the following concentrations, for comparison with monocrotaline, were carbon tetrachloride 0·01, 0·015, and 0·02 μl./ml.; thioacetamide 1, 2 and 4 μg./ml.; retrorsine 100 and 200 μg./ml.; ethionine 100, 200, 500 and 1000 μg./ml.; 2 : 4 dinitrophenol 500 and 1000 μg./ml.; dimethylaminoazobenzene 1000 μg./ml. (Table I).

TABLE I.—*Summary of the Treatments Accorded to Various Series of Test Cultures*

Series	Drug*	Concentration μg./ml.	Solvent	Time of sampling
H63/1	Monocrotaline HCl	2·5, 25, 50, 125	deionised H_2O	1, 2, 3 days
2	,,	125, 250, 500	,,	3 and 5 days
3	,,	250	,,	3 to 7 days
4	,,	1·0	,,	weekly for 15 weeks
5	,,	500	,,	,, ,, 8 ,,
6	Carbon tetrachloride	†0·01, 0·015, 0·02	Earles saline	3 to 7 days
7	Thioacetamide	1, 2, 4	deionised H_2O	,,
8	Retrorsine	100, 200	,,	,,
9	Ethionine	100, 200, 500, 1000	,,	,,
10	,,	1000	,,	weekly for 5 weeks
11	2 : 4 dinitrophenol	500, 1000	,,	3 to 7 days
12	Dimethylaminoazobenzene	1000	absolute ethanol	,,

* These substances were added in a volume of 0·1 ml. of solution per 10 ml. of medium, with the exception of carbon tetrachloride which was added as a saturated solution in Earle's saline in volumes of 0·2, 0·3, and 0·4 ml. respectively.

† Concentration measured in μl./ml.

In the case of three long term experiments (5–15 weeks), the cultures were re-fed weekly with medium containing a fresh inoculation of monocrotaline for experiments H63/4 and H63/5 and ethionine for H63/9. Subcultures were made when overgrowth demanded, using the same trypsinizing procedure as before and the controls were always subcultured at the same time.

Changes in the appearance of the cells are well known in long-established tissue cultures and, in order to minimize errors in interpretation arising from such alterations, each set of experimental cultures was paired with a set of contemporary controls.

The coverslips with the adhering cells were removed from two test and two control cultures each day from the third to the seventh day of culture. Experiment H63/1 with monocrotaline was sampled on the first, second and third day and experiments H63/4, 5 and 9, weekly (Table I). Ether/alcohol fixed cells were stained with haematoxylin and eosin or periodic acid-Schiff, and formalin fixed material stained for fat with Sudan IV.

To obtain some indication of the possible specificity of the effects of the agents used on liver cells grown for a long time *in vitro*, cultures of HeLa cells were treated for comparison, in the same manner as the liver cultures of series H63/3 (Table I) and incubated with 250 μg./ml. of monocrotaline.

Mitotic counts

Normal and abnormal mitoses were counted for each day in cultures of series H63/2 and 3. In each case 1000 cells were observed and the number of mitotic figures recorded.

Nuclear measurements

The nuclei of haematoxylin and eosin stained cells of series H63/1 and 2, receiving concentrations of 50 and 125 μg./ml. of monocrotaline were measured. The slide was projected on to paper at a fixed magnification and a total of 250 randomly selected nuclei from at least 10 different fields was outlined. The average of the greatest and smallest diameters measured at right angles was calculated for each nucleus. It was not possible to do this for nuclei of cells of cultures receiving higher doses of monocrotaline because of their irregular shape and the occurrence of the bizarre forms to be described later in this paper.

RESULTS AND OBSERVATIONS

Monocrotaline treated cultures

For the first three days of culture and with concentrations of up to 25 μg./ml. of monocrotaline, no visible differences could be detected between experimental and control cultures. At higher concentrations changes which increased in degree with dose and length of exposure could be observed.

These changes were first manifested as a slight but significant increase in nuclear size ($P = 0{\cdot}02$) in the three day cultures receiving 50 μg./ml. of monocrotaline. This can be demonstrated in the shift to right of average diameters in Fig. 1 when compared with controls. Although cytoplasmic area was not estimated, the cytoplasmic-nuclear ratio did not appear to have altered, suggesting a similar hypertrophy of this part of the cell. Nuclear enlargement was even more pronounced with concentrations of 125 μg./ml. of the alkaloid ($P < 0{\cdot}01$) (Fig. 1).

By the third day of culture and with monocrotaline in doses higher than 125 μg./ml., changes became more marked. The general growth pattern of evenly arranged squamous cells became increasingly disrupted and irregular and there appeared to be less cohesion between individual cells. Instead of the normally regular cell sheet (Fig. 4, 7), at three days the test cultures consisted of scattered groups and isolated cells with many pyknotic and dying individuals and much cell debris (Fig. 5, 6, 8, 9). There were also morphological changes from the usually hexagonal shape of the cells of the controls (Fig. 4, 7, 10, 20) to a number

of mixed and varied forms. These included some spindle-shaped cells, often with eccentric nuclei (Fig. 5, 6, 8, 9, 19), and others with very long attenuated cytoplasmic processes which in some cases appeared to bridge two cells (Fig. 16). Such processes were especially prominent in older cultures with high doses of monocrotaline where the cell population was very sparse. Abnormal hypertrophic cells of a very bizarre aspect appeared in great numbers and were of two types :

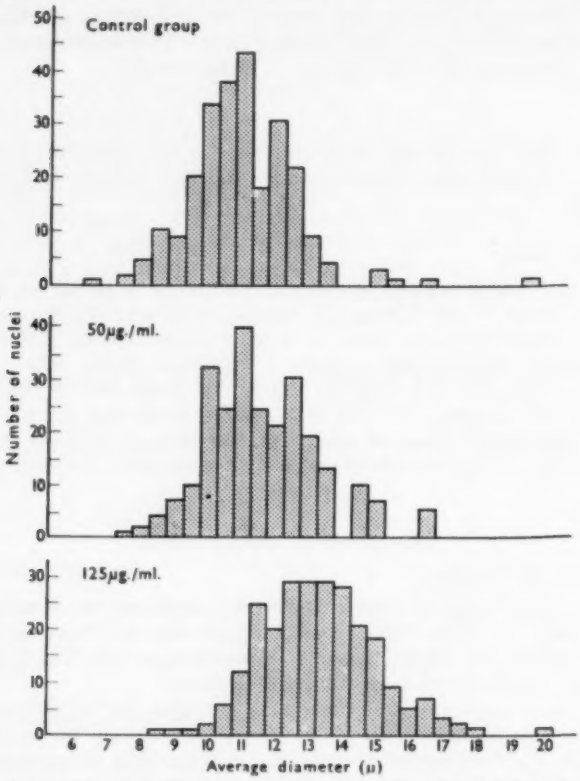


FIG. 1.—Distribution of average nuclear diameters after three days of incubation cultures of HuLi cells receiving 50 or 125 μg./ml. of monocrotaline. Measurements were made on 250 cells in each case. There is a significant shift to the right in the test cultures P = 0·02 and < 0·01 respectively) indicating nuclear hypertrophy.

(*a*) giant cells with numerous nuclei which varied considerably in size, shape and number (Fig. 5, 8, 9, 13, 17, 18, 21), and (*b*) enlarged cells with a single huge nucleus (Fig. 5, 6, 8, 9, 25). Giant cells of type (*a*) were especially numerous (Fig. 2), but both types of cell were very striking when compared with the cells of controls with their ovoid and rather regular nuclei and small well defined nucleoli (cf. figures of control and experimental cultures).

Irregular, fused and misshapen nucleoli (Fig. 13, 14, 18, 25) were a feature of

the nuclei of both kinds of enlarged cell in test cultures (we would like to term these megalocytes) and coarse, granular, deeper-staining chromatin was sometimes present (Fig. 5, 8, 14, 21, 25). The cytoplasm of the enlarged cells was often tenuous or finely granular, and sometimes contained numerous small or one or two very large vacuoles which were Schiff negative and did not contain fat (Fig. 11, 12, 17, 25). Amorphous eosinophilic bodies within vacuoles were sometimes present in the cytoplasm of haematoxylin and eosin stained test cultures (Fig. 11, 16, 19) although these were very occasionally seen among cells of the controls.

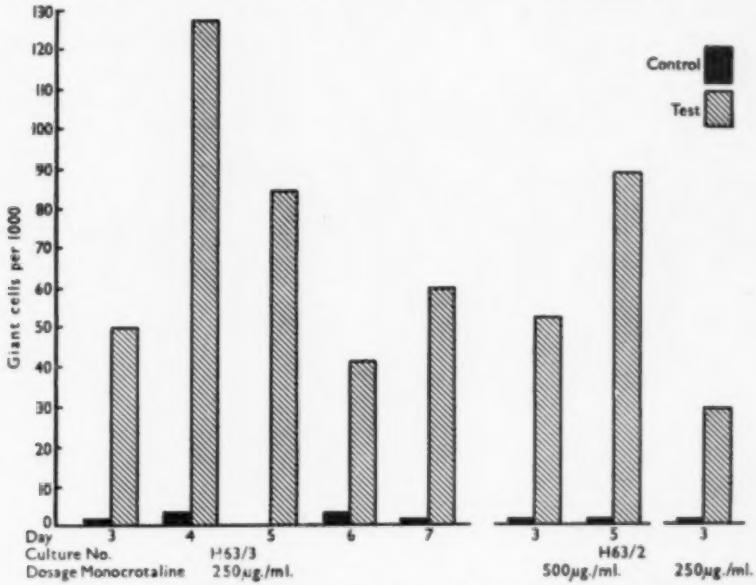


FIG. 2.—Histogram showing the increased incidence of giant multinucleated cells per 1000 cells in HuLi cultures. Cultures received 250 or 500 µg./ml. of monocrotaline with exposures of three to seven days.

The control cultures also sometimes contained enlarged cells of type *b*, but these were not as hypertrophied as in test cultures where there was a progressive increase in size with monocrotaline, even before the appearance of the more grossly abnormal cells. The few multinucleated cells which appeared in the controls (Fig. 2) did not exhibit the degree of nuclear variability nor reach the size of those of the test cultures.

Mitotic counts and growth rate

The rate of growth as judged by the number of mitoses was followed principally in a series (H63/3) where cultures were given 250 µg./ml. of monocrotaline and sampled daily from the third to the seventh day of incubation.

From Fig. 3 it can be seen that the growth rate of the controls was fairly high at three days and declined with age as the available space became colonized.

The picture presented by the test cultures was more erratic. The number of mitoses did not diminish as expected. On further examination (Fig. 3) a large number of divisions—in some cases nearly 50 per cent of the total—appeared abnormal in some way, with atypical spindles which were often multipolar or deformed. Heteroploidy was common and sticky or clumped chromosomes often seen (Fig. 14, 15, 22, 23, 24). Abnormal mitoses were rarely found in controls (Fig. 3). Some cells of test cultures appeared to be dividing but without evidence

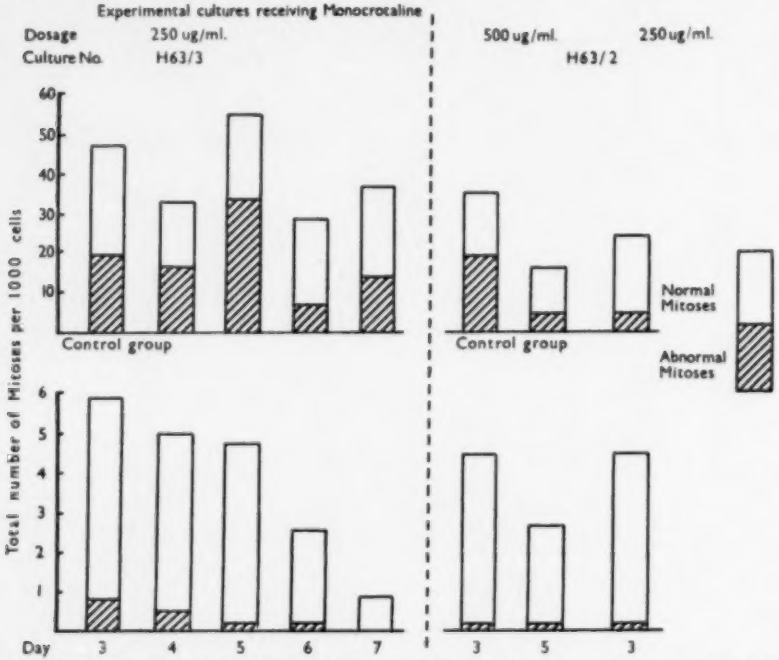


FIG. 3.—Total, normal and abnormal mitoses per 1000 cells in HuLi cultures receiving 250 or 500 μg./ml. of monocrotaline. The height of each column represents the total mitoses, while normal and abnormal divisions are shown by the unshaded and crosshatched areas respectively.

of a mitotic spindle (Fig. 13) and no mitosis was ever observed in a giant cell although several of these had constrictions of the cytoplasm (Fig. 13), cytoplasmic bridging (Fig. 16) and nuclear indentations (Fig. 18).

Long term experiments with monocrotaline

The experiments were performed to investigate the action of repeated high and low doses of monocrotaline. Cultures of H63/4 receiving 1 μg./ml. per week were not visibly different from controls after 15 weeks. At this time the cultures were accidentally lost. In the second experiment H63/5 with the high dose of monocrotaline of 500 μg./ml. per week, the bizarre changes rapidly appeared,

the cultures declined very quickly and died. After eight weeks only a few pyknotic cells remained clinging to the glass vessel.

Experiments with other hepatotoxic agents

Of the substances, in the doses used (Table I), only retrorsine (another pyrrolizidine alkaloid), 2 : 4 dinitrophenol and dimethylaminoazobenzene produced megalocytes which resembled those induced by monocrotaline. Retrorsine appeared to affect HuLi to the same degree as monocrotaline, but abnormal cells were few in cultures receiving the other two substances. Hypertrophied mononuclear cells were not especially obvious after treatment with 2 : 4 dinitrophenol or dimethylaminoazobenzene.

Experiments with HeLa cells

As in controls of HuLi, control HeLa cultures also contained a few multinuclear and enlarged cells, but the addition of monocrotaline did not seem to be remarkable in its effects.

DISCUSSION

Greatly enlarged cells, " megalocytes," have been observed in the livers of rats treated with some of the pyrrolizidine alkaloids including retrorsine (in this laboratory), and monocrotaline (Harris, Anderson and Chen, 1942) and in the livers of domestic animals and man after ingestion of plants containing these substances (Bull, 1955 ; Hill and Martin, 1958). It is interesting therefore, that the first change to be noted in these cultures was a slight increase in cell and nuclear size which became more marked with prolonged exposure to the drug.

Two types of enlarged cell were found in the test cultures in these experiments, the most prevalent being the giant cell containing numerous nuclei. Cells of this type have not been described *in vivo* either after experimental administration of pyrrolizidine alkaloids or in the naturally occurring disease. Multinucleate cells, however, have been observed in many established cultures of both malignant and non-malignant origin after long periods *in vitro*. Jordan (1956) described cells which possessed many nuclei in cultures from normal human nasal mucosa and Lelli, Balducci, Gori and Bondi (1957) noted them in strains of normal human liver, KB and HeLa cultures. Berman, Stulberg and Ruddle (1957) also mention multinucleate cells in cultures from many sources, both cancerous and normal. All these authors record multinucleate cells in fairly small numbers which agrees with present observations on controls where the incidence was about 0–0·2 per cent. Such cells in controls did not show the degree of variability in number of nuclei and the increase in cell size apparent in test cultures. It is possible therefore to regard enlarged cells with both one and several nuclei as having undergone some hyperplastic change which has been induced by monocrotaline.

Monocrotaline (and also retrorsine) in this instance, probably did not act solely on existing multinuclear cells. It does not seem possible to explain the striking increase in number unless monocrotaline has an effect on cell division at least *in vitro*. The increased prevalence of aberrant and multipolar mitoses in experimental cultures is an indication of some interference with this process. Pomerat, Kent and Logie (1957) observed that similar abnormalities increased

and also larger numbers of giant cells appeared in cultures of nine different strains, including normal human liver, after exposure to irradiation. Heteroploidy had been demonstrated in these cultures and Pomerat *et al.* (1957) attribute multinuclear giant cell formation to this, suggesting that they are the result of several mitoses within the same cell where subsequent cytoplasmic division fails to occur.

EXPLANATION OF PLATES

FIG. 4.—Three day control culture, showing sheet of regular and rather cohesive cells with several mitotic figures. Cf. Fig. 5 and 6. H. and E. ×85.

FIG. 5.—Three day culture with 250 μg./ml. of monocrotaline. Disrupted and less cohesive cell sheet showing slight increase in cell and nuclear size compared with control in Fig. 4. Note bizarre forms, spindle-shaped cells and mono and multinuclear giant cells. H. and E. ×85.

FIG. 6.—Three day culture with 500 μg./ml. of monocrotaline. As Fig. 5 but showing more exaggerated effect. H. and E. ×85.

FIG. 7.—Five day control culture. Squamously arranged dense sheet of cells covering most of available space with only slight variations in cell size. H. and E. ×85.

FIG. 8.—Five day culture with 250 μg./ml. of monocrotaline. Poorly populated culture with an increased proportion of bizarre cells when compared with Fig. 5 and 6. Note fine cytoplasmic extensions and frequent vacuolation. H. and E. ×85.

FIG. 9.—Five day culture with 500 μg./ml. of monocrotaline. As Fig. 8. H. and E. ×85.

FIG. 10.—Three day control culture, for comparison with Fig. 11–19. H. and E. ×340.

FIG. 11.—Three day culture with 250 μg./ml. of monocrotaline. Binucleate cell containing an eosinophilic body within a vacuole (→). An adjacent multinuclear cell has finely vacuolated cytoplasm. H. and E. ×340.

FIG. 12.—Three day culture with 250 μg./ml. of monocrotaline. The centre cell contains one huge vacuole which has pushed aside the nuclei. H. and E. ×340.

FIG. 13.—Three day cultures with 250 μg./ml. of monocrotaline. A multinucleated cell with a central constriction (→) suggesting amitotic division. Note the irregularly shaped nucleoli in surrounding cells. H. and E. ×340.

FIG. 14.—Three day culture with 250 μg./ml. of monocrotaline. Many nuclei show fused nucleoli including the nucleus (a) which also contains hyperchromatic material concentrated at the edges of the nuclear membrane. (b) A quadripolar mitosis nearing completion. H. and E. ×340.

FIG. 15.—Three day culture with 500 μg./ml. of monocrotaline. Group of abnormal mitoses showing "sticky" and clumped chromosomes. H. and E. ×340.

FIG. 16.—Three day culture with 500 μg./ml. of monocrotaline. Bridging of cytoplasm (→) between two cells. Note eosinophilic bodies. H. and E. ×340.

FIG. 17.—Three day culture with 500 μg./ml. of monocrotaline. An enlarged binucleated cell with vacuolated cytoplasm. Cf. size of the nuclei with controls in Fig. 10. H. and E. ×340.

FIG. 18.—Three day culture with 500 μg./ml. of monocrotaline. Two multinucleated giant cells. Cell (*a*) hyperchromatic nuclear material, irregular nucleoli and an indented nucleus (→). Cell (*b*) a large number of extremely small nuclei. H. and E. ×340.

FIG. 19.—Three day culture with 500 μg./ml. of monocrotaline. Spindle-shaped cells with eccentric nuclei. H. and E. ×340.

FIG. 20.—Five day control culture for comparison with Fig. 21–25. Small cells of equal size with ovoid nuclei and small nucleoli. H. and E. ×340.

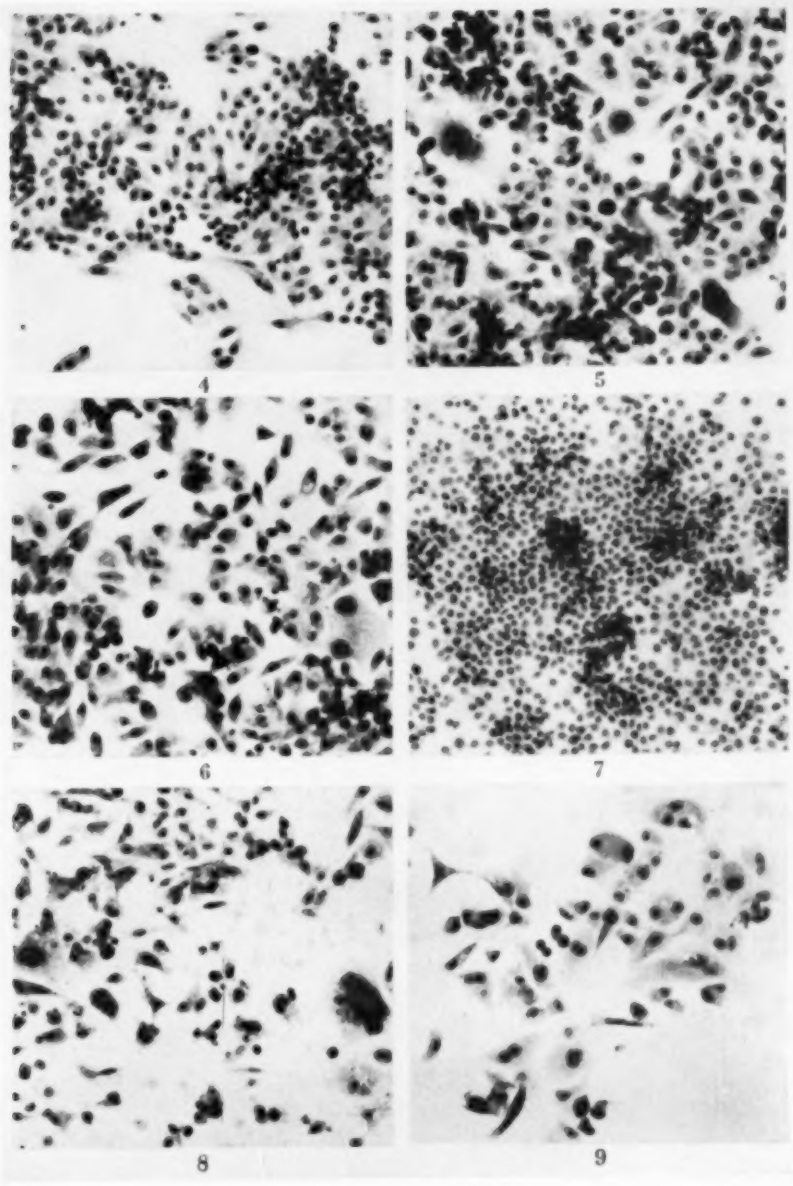
FIG. 21.—Five day culture with 250 μg./ml. of monocrotaline. Giant cell with numerous nuclei of varying size. Note darker staining nuclei of surrounding cells. H. and E. ×340.

FIG. 22.—Five day culture with 250 μg./ml. of monocrotaline. Quadripolar mitosis in metaphase. An adjacent cell also shows abnormal mitosis. H. and E. ×340.

FIG. 23.—Five day culture with 250 μg./ml. of monocrotaline. An enlarged mononuclear cell in abnormal mitosis at an earlier stage (metaphase) than that of Fig. 24. The mononuclear origin of the chromosomes is obvious here. H. and E. ×340.

FIG. 24.—Five day culture with 250 μg./ml. of monocrotaline. (*a*) Greatly hypertrophied mononuclear cell showing abnormally high number of chromosomes with five main condensations. Scattered, isolated chromosomes can also be seen. (*b*) Cell in late telophase. (*c*) Cell in metaphase, note misplaced chromosome. H. and E. ×340.

FIG. 25.—Five day culture with 250 μg./ml. of monocrotaline. Mononuclear giant cell with hyperchromatic nuclear material and abnormal nucleoli. Cf. size of cells of control in Fig. 20. H. and E. ×340.



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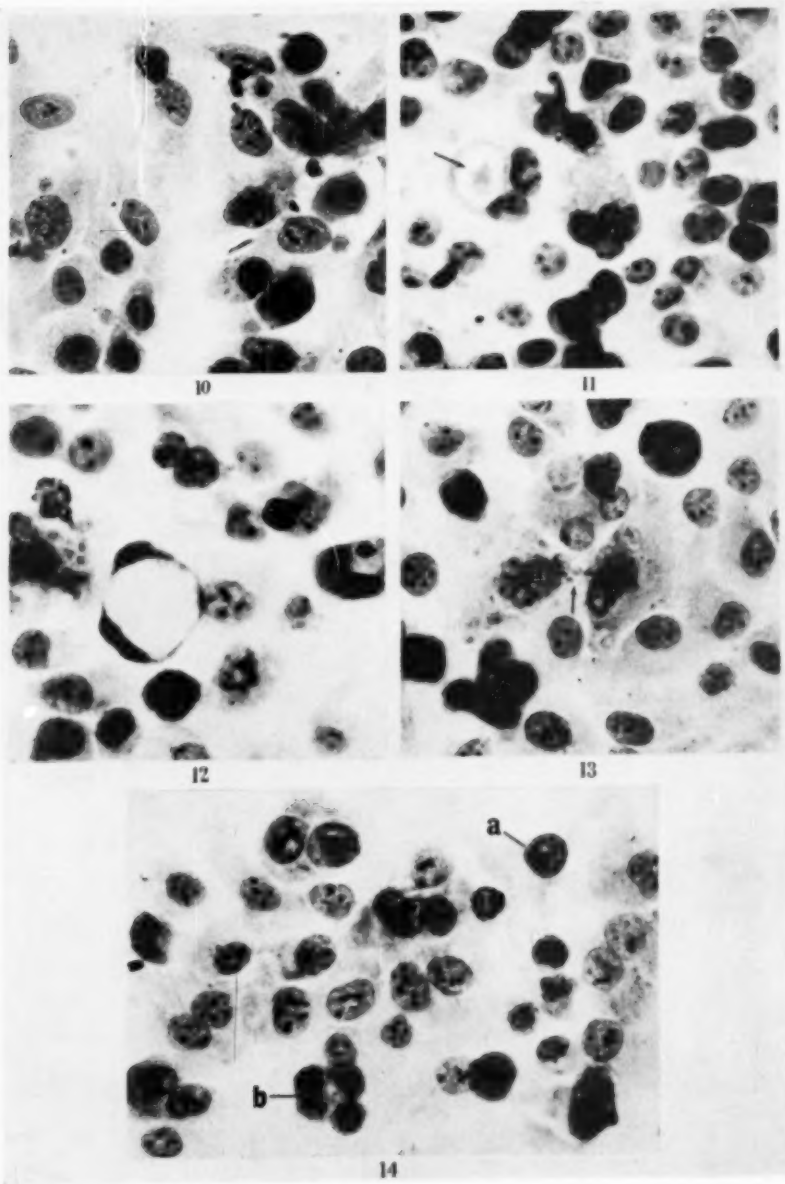

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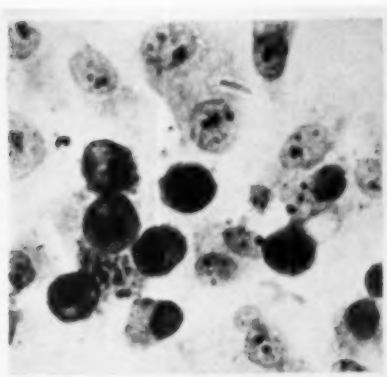

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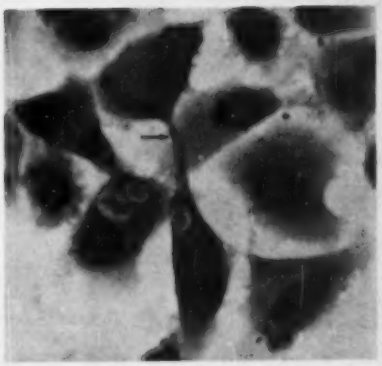

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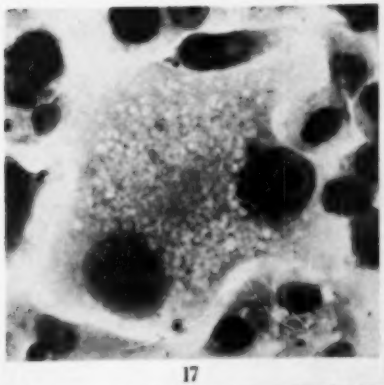

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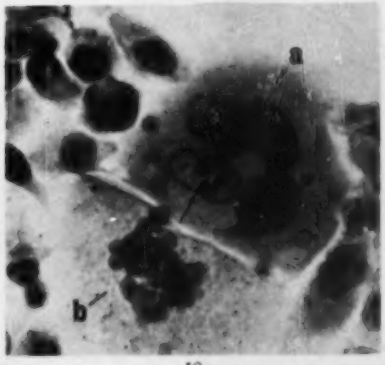

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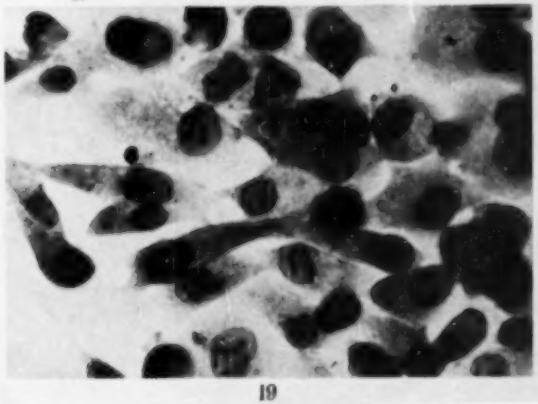

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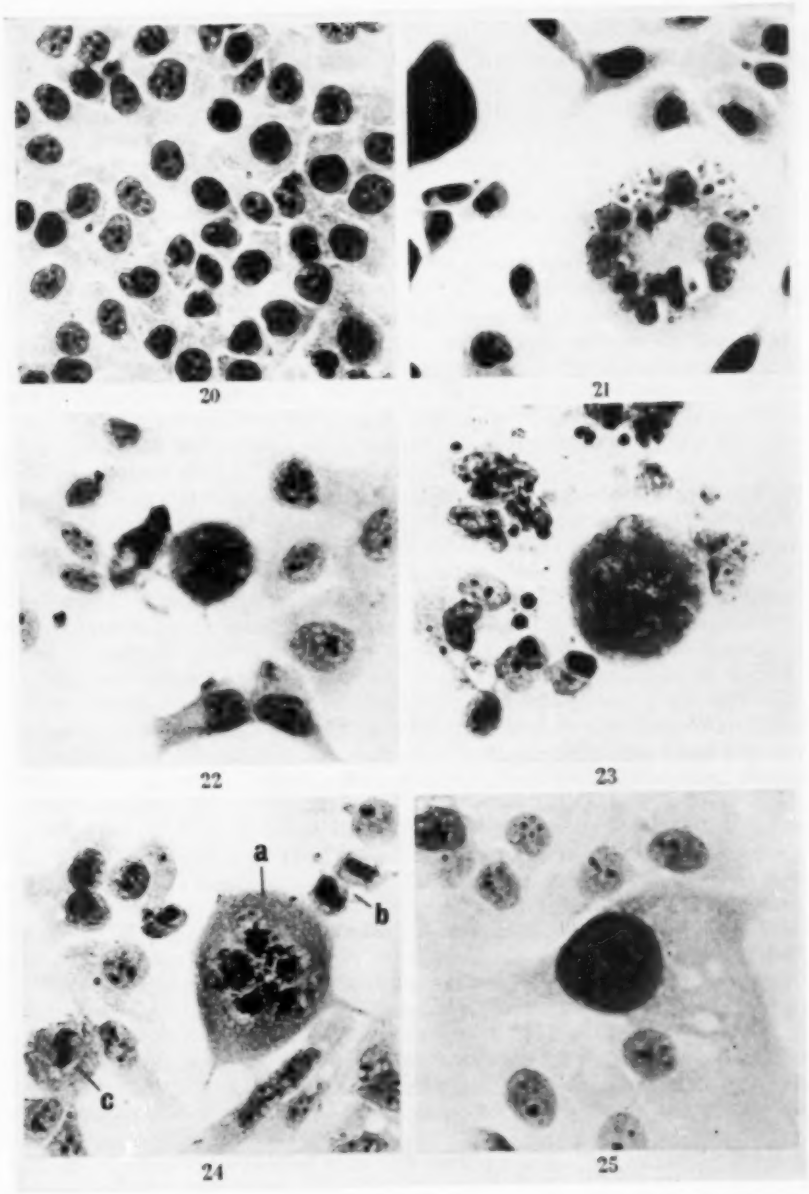

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Heteroploidy and many multipolar mitotic figures were frequently observed in the test cultures of the present study.

A different explanation of the multinuclear condition has been put forward by Bucher (1958) who discussed the origin of the binucleate cells in his cultures of osteoblasts and fibroblasts and concluded that such a condition arose by amitotic division. According to this author, a rise in the numbers of binucleate (and therefore presumably multinucleate) cells is an indication of amitotic division which has been followed by a failure of cytoplasmic division. It may be that the frequent aberrant mitoses observed in these tests indicate abnormalities in chromosome numbers. Imbalance of chromatin material may then be the cause of mitotic failure. Although abnormal numbers of chromosomes were observed in some cells, chromosome studies have yet to be performed on monocrotaline treated cultures. In favour of the existence of amitosis in our cultures, is the fact that mitoses were never observed in multinucleate giant cells or in the numerous binucleate cells, while there were often constrictions of the cytoplasm and indentation and budding of the nuclei, suggesting that some sort of division was in progress. While some multinuclear giant cells may have arisen by mitosis in the fashion described by Pomerat *et al.* (1957), it is probable that amitotic division occurred in established multinuclear cells.

Another factor in favour of amitotic activity, is that the total number of mitoses of test cultures compared with controls, did not differ greatly except toward the seventh day of culture. The reason for this difference may be that at this time the cells of the control cultures had colonized most of the available space and growth had slowed to a minimum maintenance rate, but in test cultures there was much cell death leaving a considerable area of unpopulated substratum. If this explanation of the minor difference in rate of mitoses is acceptable, amitotic activity must have occurred in what would normally be the resting cells at the time of sampling to produce the enormous numbers of multinuclear giant cells. Monocrotaline (and retrorsine) thus can be assumed to stimulate amitotic division.

The characteristics of the cells described in experimental test cultures such as increase in size, pleomorphism, the great variation in nuclear size, shape and number per cell, the irregularities of the nucleoli and the coarseness of the chromatin material are features which frequently have been ascribed to neoplastic cells. While such changes were not entirely absent in the controls and have often been described in long established cell lines, the marked rapid increase in these features after exposure to monocrotaline may be stressed. A similar increase was noticed by Pomerat *et al.* (1957) after irradiation of their cultures. Moore, Southam and Steinberg (1956) have injected suspensions of cells of normal origin, which developed such characteristics after prolonged culture *in vitro*, into suitably prepared animal and human hosts. These authors found that palpable nodules of a histologically malignant appearance developed. It is also significant that HeLa cells which are neoplastic in origin were unaffected by doses of monocrotaline which produced such startling changes in the liver cells. It is possible therefore that monocrotaline is capable of producing a truly neoplastic change at least *in vitro* in a tissue of normal origin.

Finally it should be added that the findings presented must be interpreted with reservation when applied to possible reactions *in vivo*, since the metabolism of an established strain of cells such as HuLi must surely have altered from its original state.

SUMMARY

1. A strain of human embryo liver cells was used to investigate the action of the pyrrolizidine alkaloid, monocrotaline, other known hepatotoxic agents being used for comparison.

2. A strain of HeLa cells was treated with monocrotaline for comparison with liver cells.

3. The first effect of monocrotaline on liver cells, noted at three days, with doses of 50 and 125 μg./ml., was a significant increase in nuclear size. With higher doses of the drug, cells became less cohesive and increasing numbers of bizarre cells appeared. The latter included two types of megalocyte : (*a*) mononucleated and (*b*) multinucleated cells. The multinucleated cells contained varying numbers of unequal nuclei and comprised about 3–13 per cent of the total population compared with only 0–0·2 per cent in controls.

4. Abnormal mitoses were much more frequent in test cultures.

5. There was some evidence to show that amitotic divisions occurred in monocrotaline treated cultures.

6. Features similar to those seen in neoplastic cells were also seen to increase in cultures receiving monocrotaline.

7. Of the other drugs used, only retrorsine, 2 : 4 dinitrophenol and dimethylaminoazobenzene produced changes similar to those described for monocrotaline.

8. HeLa cells remained unaffected by monocrotaline.

9. The findings suggest that monocrotaline induces *in vitro*, amitotic division and possibly neoplastic changes in embryonic liver cells.

The authors would like to thank the British Empire Cancer Campaign for the generous grant-in-aid which made this work possible and to acknowledge the help of Miss A. Demery, M.A., Miss H. Haestier, Miss U. Hopkins, Mrs. A. Birbeck and Mr. R. R. Phillips in the preparation of this paper.

REFERENCES

Berman, L., Stulberg, C. S. and Ruddle, F. H.—(1957) *Cancer Res.*, **17**, 668.
Berry, D. M. and Bras, G.—(1957) *N. Amer. Vet.*, **38**, 323.
Bras, G. and Hill, K. R.—(1956) *Lancet*, i, 960.
Bucher, O.—(1958) *Z. mikr.-anat. Forsch.*, **64**, 174.
Bull, L. B.—(1955) *Aust. vet. J.*, **31**, 33.
Campbell, J. G.—(1956) *Proc. Roy. Soc. Edinb.* B., **66**, 111.
Cook, J. W., Duffy, E. and Schoental, R.—(1950) *Brit. J. Cancer*, **4**, 405.
Harris, P. N., Anderson, R. C. and Chen, K. K.—(1942) *J. Pharmacol.*, **75**, 83.
Hill, K. R.—(1959) *Trans. R. Soc. trop. Med. Hyg.*, **53**, 217.
Idem and Martin, H. M.—(1958) *Brit. vet. J.*, **114**, 345.
Jordan, W. S.—(1956) *Proc. Soc. exp. Biol. N.Y.*, **92**, 867.
Lelli, G., Balducci, D., Gori, G. B. and Bondi, M.—(1957) *R.C. Ist. sup. Sanit.*, **20**, 1113.
Moore, A. E., Southam, C. M. and Steinberg, S. S.—(1956) *Science*, **124**, 127.
Pomerat, C. M., Kent, S. P. and Logie, L. C.—(1957) *Z. Zellforsch.*, **47**, 158.
Schoental, R. and Head, M. A.—(1955) *Brit. J. Cancer*, **9**, 229.
Idem, Head, M. A. and Peacock, P. R.—(1954) *Ibid.*, **8**, 458.
Idem and Magee, P. N.—(1959) *Acta Un. int. Cancr.*, **15**, 212.
Westwood, J. C. N., McPherson, I. A. and Titmuss, D. H. J.—(1957) *Brit. J. exp. Path.* **38**, 138.

THE EFFECT OF TRAUMA ON THE MELANOTIC TUMOURS OF THE HAMSTER

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MECHANICAL trauma has frequently been accused of producing tumours or converting benign tumours into malignant ones. An outstanding example of this is the malignant melanoma of man which many believe can arise as a result of trauma to a mole (naevus) or even normal skin (Hewer, 1935; Traub and Kiel, 1940; Webster, *et al.*, 1944; Willis, 1948). Other workers however (Slaughter, 1948; Davis, 1955; Pack, 1957; Belisario, 1959) do not accept this thesis. They believe that as yet there is no authenticated case of a mole having become malignant as a result of trauma and that the local appearance of a malignant melanoma after incomplete removal of a supposed benign naevus indicates that the lesion was already malignant before removal.

A parallel experimental investigation of this problem is difficult, for naturally occurring melanotic naevi are not to be found in common laboratory animals. However, numerous melanotic tumours are readily produced by the repeated application of 9 : 10-dimethyl-1 : 2 benzanthracene (DMBA) to hamster skin (Della Porta *et al.*, 1956; Ghadially, 1959; Ghadially and Barker, 1960). The majority of these are benign lesions and in that respect at least they are similar to the common mole of man. It seemed interesting therefore to investigate whether mechanical trauma could convert such benign melanotic tumours into malignant ones. It is realised that such a study cannot directly bear upon the controversy regarding the effect of trauma to human naevi, but nevertheless it is important to find out if an experimental situation can be created in which trauma turns a benign melanotic lesion into a malignant one.

MATERIALS AND METHODS

Brown and white hamsters weighing approximately 90 g. were painted once a week on the left flank with an 0·2 per cent solution of DMBA in acetone for a period of 25 weeks. After a rest period of approximately one month animals bearing melanotic tumours were segregated. The tumour-bearing flank of every animal was photographed and a chart indicating the size and position of each tumour around the costo-vertebral spot prepared for future reference and for plotting the course of events. Three separate experiments were performed as follows.

Experiment 1

Twenty-five brown hamsters bearing melanotic tumours ranging in size from $\frac{1}{2}$ mm. to 3 mm. were divided into two groups: an experimental group of 15 animals bearing 48 tumours between them and a control group of 10 showing 35 tumours, of similar size and distribution.

The 48 tumours on the experimental animals were traumatized by pricking with a needle (0·5 mm. diameter) right through the tumour and the full thickness of the skin. With the needle in this position the tumour was further traumatized by squeezing it on to the needle with a pair of forceps. This procedure was repeated at weekly intervals on 6 occasions. The animals were observed for a further period of 4 months.

Experiment 2

Five brown hamsters bearing eleven tumours varying in size from ½ to 3 mm. were traumatized by cutting across them with a pair of scissors. The cut extended right through the skin into the subcutaneous tissues. This procedure was repeated after a fortnight. Another 5 animals bearing 14 tumours served as controls. These animals were observed for a further period of 3 months.

Experiment 3

Seven white and five brown hamsters bearing 30 melanotic and hypomelanotic tumours ranging in size from 3 mm. to 14 mm. were available for study. (Smaller tumours were also present in these animals but they were ignored for the purpose of this experiment). Each of these animals also bore confluent ulcerated keratoacanthomas or squamous carcinomas. These tumours were excised and the margins of the wound everted so as to expose the under surface of the adjacent melanotic tumours. The base of seven melanotic and hypomelanotic tumours exposed in this fashion was shaved off so as to liverate the tumour from its ensheathing capsule and bring it into free contact with underlying tissues. Another seven tumours were traumatized by placing a chrominized catgut ligature in their substance and a further seven were damaged by incorporation into the margin of the wound created by the excision of the epithelial tumours. Nine untraumatized tumours on the same animals served as controls. These animals were observed for a period of 4 months after the infliction of the trauma.

RESULTS

In none of the traumatized tumours did we observe any rapid increase in size nor did they show obvious malignant transformation by infiltrating the deeper tissues or producing metastases in distant organs. Indeed some of the small tumours traumatized in Experiments 1 and 2 became paler and smaller and at times disappeared completely from sight. This phenomenon was also seen to occur occasionally in small untraumatized tumours in control animals but it was more frequent in the traumatized tumours. Fig. 1 shows a traumatized tumour which appeared to become paler. There is an obvious paucity of pigmented

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EXPLANATION OF PLATE

FIG. 1.—Traumatized melanotic tumour showing paucity of pigmented tumour cells in the superficial part of the tumour. H. and E. × 155.

FIG. 2.—Melanotic tumour fragmented by trauma. H. and E. ×255.

FIG. 3.—Oedematous vascular scar in traumatized tumour (pale areas in photograph). Masson's trichrome ×155.

FIG. 4.—High power view from Fig. 3. Note oedematous collagen in top left-hand corner, and blood vessels flanked by melanin-containing cells. H. and E. ×620.

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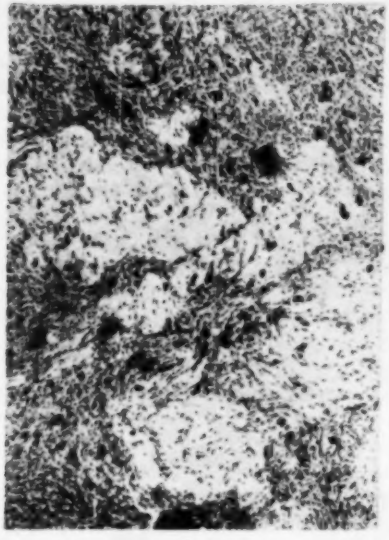

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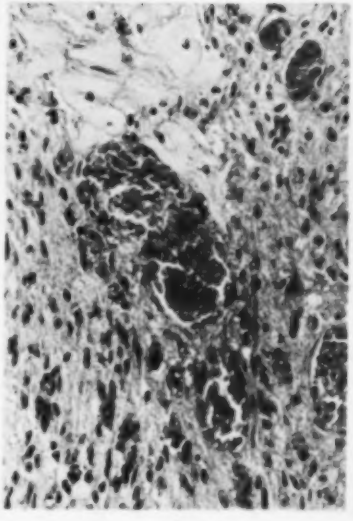

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Ghadially, Illman and Barker.

tumour cells particularly in the superficial parts. Here replacement fibrosis, and foci of inflammatory cells were detected under higher magnification. Fig. 2 shows another traumatized tumour which has become fragmented as a result of multiple injuries. In both these tumours (Fig. 1 and 2) the residual tumour cells do not show any heightened mitotic activity or exaggerated cellular pleomorphism and anaplasia, nor is there any evidence of unusual or marked infiltrative activity.

Fig. 3 shows a large hypo-melanotic tumour traumatized by insertion of a ligature which later dropped off. A vascular oedematous scar has formed in this tumour. This tumour is markedly hypomelanotic, yet around the scar there are many collections of cells containing abundant melanin (Fig. 4). It would appear that trauma has stimulated local pigment production and/or accumulation. This feature was observed in other hypomelanotic or virtually amelanotic tumours also.

DISCUSSION

Our results show that single or repeated mechanical trauma has failed to effect an indubitable malignant transformation of a melanotic tumour in the hamster. This is in keeping with current opinion that trauma does not precipitate malignant change in human moles (Belisario, 1959).

It is extremely difficult to evaluate the role of trauma in tumour production or in the transformation of benign tumours to malignant ones. Species difference seems to play a dominant role. Mechanical trauma to carcinogen-treated skin of the rabbit is readily followed by the appearance of tumours, but in the mouse rarely can tumours be produced by this method (Berenblum, 1954).

In experiment 3 a large deep wound was created in each animal by the operative technique involved. This did not lead to the production of any new epithelial or melanotic tumours around the margins of the wounds, though a type III keratoacanthoma situated near the margin of a wound seemed to show a temporary rapid increase in size. The situation in man is difficult to assess, but reports of a variety of cutaneous tumours occurring at the site of injury may be found in the literature (Belisario, 1959).

The increased production of melanin in injured hypomelanotic melanomas is of some biological interest. It is well known that hyperpigmentation may occur around traumatic scars, particularly those produced by burns. Such hyperpigmentation is also seen around insect bites and after the application of carcinogenic or non-carcinogenic irritants. The increased pigmentation seen in traumatized hypomelanotic melanomas is probably no more than a reflection of the property of the melanocyte to respond by increased melanin production and/or storage when situated in a traumatized area.

Another interesting observation made by us was that traumatized and non-traumatized melanotic tumours may occasionally disappear from sight. It must be pointed out that this is only true of very small lesions below 1 mm. in size. It is interesting to recall that many of the benign epithelial tumours produced by painting rodent skin with carcinogens also regress and disappear. In this case it is a true regression (Ghadially, 1959) for the tumour cells disappear and at the end of the process only a small scar remains. We do not know whether this is true in the case of the melanotic tumour. It is conceivable that the tumour may disappear

from sight by discharging its melanin rather than by a loss of cells. If that is the case it cannot be considered a true regression.

SUMMARY

Single or repeated trauma to carcinogen induced melanotic tumours of the hamster did not produce malignant change in these tumours. In the majority of cases these tumours were not materially affected by trauma, but in some instances small tumours disappeared from sight. In larger hypomelanotic tumours a vascular scar was formed, surrounded by cells containing abundant melanin.

We are indebted to Mr. T. L. Platts and Miss S. D. Wall for photomicrographs, to Mr. J. N. Carver and Miss J. A. Osborne for technical assistance. This work was supported by grants to one of us (F. N. G.) from the British Empire Cancer Campaign and the University of Sheffield Medical Research Fund.

REFERENCES

Belisario, J.—(1959) ' Cancer of the skin '. London (Butterworths), p. 190 and p. 20.
Berenblum, I.—(1954) *Cancer Res.*, **14**, 471.
Davis, J.—(1955) *Postgrad. Med.*, **18**, 138.
Della Porta G., Rappaport, H., Saffiotti, V. and Shubik, P.—(1956) *Arch. Path.*, **61**, 305.
Ghadially, F. N.—(1959) *J. Path. Bact.*, **77**, 277.
Idem and Barker, J. F.—(1960) *Ibid.*, **79**, 263.
Hewer, T. F.—(1935) *Ibid.*, **41**, 473.
Pack, G. T.—(1957) *Virginia med. (Semi-)Mon.*, **84**, 111.
Slaughter, D. P.—(1948) *Surg. Clin. N. Amer.*, **28**, 69.
Traub, E. F. and Kiel, H.—(1940) *Arch. Derm. Syph., Chicago*, **41**, 214.
Webster, J. P., Stevenson, T. W. and Stout, A. P.—(1944) *Surg. Clin. N. Amer.*, **24**, 319.
Willis, R. A.—(1948) ' Pathology of Tumours', London (Butterworths), p. 906.

TUMOUR PROMOTION BY THE NEUTRAL FRACTION OF CIGARETTE SMOKE

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IT is known that the incidence of lung cancer amongst heavy cigarette smokers is higher in urban than in rural districts. While this might be attributed solely to exposure to the carcinogenic substances present in the urban atmosphere (e.g. particularly smoke from coal fires, also diesel fumes and car exhaust fumes (Kotin, 1956)), there is also the possibility of a co-carcinogenic action between cigarette smoke and city smoke. The following series of experiments was designed to test this hypothesis. Since the experiments were begun three years ago, Gellhorn (1958), Roe, Salaman and Cohen (1959) and Wynder and Hoffman (1960) have confirmed the presence of incomplete carcinogens in cigarette smoke as foreshadowed by Hamer and Woodhouse (1956) and Gwynn and Salaman (1956).

MATERIAL AND METHODS

One hundred and fifty-six C57BL mice (68 females, 88 males) were divided into 7 groups for the purposes of treatment. Aged between 3 and 6 weeks at the beginning of the experiment, the mice were allowed to live to the end of their normal lives or, in the case of those with skin tumours, they were killed when the tumours became large or frankly malignant. All skin tumours except the smallest papillomata were sectioned and examined histologically. A tumour was judged to be malignant when the panniculus carnosus was invaded. Tumours were classed as " probably malignant " when the tumour cells had not yet reached the muscle although other signs of malignancy were present.

The test substances in solution were applied with two strokes of a No. 4 paint-brush to the skin in the interscapular region. There were three substances: fraction " C " from city smoke, a known carcinogenic material (Clemo, Miller and Pybus, 1955), applied as a 1·0 per cent solution in benzene; croton oil, a known tumour promoter, applied as a 0·5 per cent solution in acetone; and the neutral fraction of cigarette smoke applied as a 10 per cent solution in benzene. This last fraction was extracted from the whole tar from cigarette smoke as described by Clemo (1958) and approximately 6·4 mg. was applied to each mouse at each painting.

The 7 groups in the experiment received the following treatments:

Group I.—Twenty-one mice (9 females, 12 males) were painted 3 times a week, for 2 weeks only, with fraction " C ". They received no further treatment.

Group II.—Twenty-one mice (6 females, 15 males) were painted with fraction " C" as in Group I. After an interval of 3 weeks they were painted with neutral fraction 3 times a week till death.

Group III.—Twenty-four mice (15 females, 9 males) were painted with fraction " C " as in Group I. After an interval of 3 weeks they were painted with croton oil twice weekly until death.

Group IV.—Twenty-two mice (8 females, 14 males) were painted 3 times a week, for 2 weeks only, with neutral fraction. They received no further treatment.

Group V.—Twenty-one mice (6 females, 15 males) were painted with neutral fraction as in Group IV. After an interval of 3 weeks they were painted with fraction " C " 3 times a week until death.

Group VI.—Twenty-five mice (11 females, 14 males) were painted with neutral fraction as in Group IV. After an interval of 3 weeks, they were painted with croton oil twice weekly until death.

Group VII.—Twenty-three mice (13 females, 10 males) were painted with croton oil twice weekly until death.

RESULTS

The results are summarised in Table I.

Group I.—The dose and duration of painting with fraction " C " were arbitrary. It was known to produce many skin tumours in C57BL mice when applied throughout life (Clemo and Miller, 1957). From the present experiment it was evident that a minimal carcinogenic dose had been given. Two males (aged 19 and 29·5 months) developed 3 small papillomata, 2 of which, at the site of painting, were superficial, while in the third (slightly larger and on the abdominal surface) the epithelium had not yet reached the panniculus carnosus. One female developed a rapidly growing spindle-celled subcutaneous sarcoma over the left scapula, at the age of 20 months. The non-tumour mice all lived well into tumour age, 4 dying with leukaemia, 2 with hepatomata and one with leukaemia plus hepatoma.

Group II.—Three males developed epitheliomata (one per mouse) at the site of painting, all growing steadily so that the mice had to be killed 3 to 4 months after the first appearance of the lesions ; 7 papillomata appeared in 5 other males, all at the site of painting. In 4 females there were 8 skin tumours (one mouse had 4 and in another mouse 2 coalesced to form one) ; of these, 5 were innocent papillomata but 3 were classified as " probably malignant ". Non-tumour mice lived well into tumour age ; one female died at 11 months of leukaemia, the other at 15·5 months of pneumonia, while gross kidney disease caused the deaths of the 7 non-tumour males.

Group III.—The maximum total number of skin tumours in the 9 tumour females was 14, but 4 disappeared before death, leaving a final total of 10, the greatest individual number being 4 ; of these 10, one grew slowly but steadily to become " probably malignant " at the time of death 8 months later, the rest remaining small. The three tumours in males (one each) were all small. The non-tumour mice were all of tumour age and the majority died with grossly diseased kidneys.

Group IV.—In this group 2 male mice each developed one small skin papilloma ; one of these, in the lumbar region, disappeared before death ; the second was in the centre of the abdominal surface. The remaining mice died at tumour age without skin tumours but 3 had leukaemia, one a very large lung tumour (rare in this strain), one had haemangioma of the spleen, one a hepatoma and the rest had diseased kidneys.

TABLE I.—*The Tumour-promoting Effect of the Neutral Fraction of Cigarette Smoke, after Tumour Initiation by Fraction "C" from City Smoke*

Group (1)	Treatment (2)	Number of mice at risk (3)	Tumour mice: Number (4)	Tumour mice: Per cent (5)	Number of skin tumours: Total: Maximum (6)	Number of skin tumours: Total: Final (7)	Number of skin tumours: Probably malignant (8)	Number of skin tumours: Malignant (9)	Age of mice in months: Tumour: At tumour appearance: Av. (10)	At tumour appearance: Range (10)	Tumour: At death: Av. (11)	At death: Range (11)	Non-tumour At death: Av. (12)	Non-tumour At death: Range (12)
I	C + *nil*	21	3	14·3	4	4	0	1	22·8	(19·0–29·5)	26·5	(31·0–30·5)	25·6	(21·0–31·0)
II	C + neutral	21	12	57·1	18[1]	17	3	3	20·2	(9·5–28·0)	21·9	(13·5–28·5)	20·3	(11·0–28·0)
III	C + croton oil	24	12	50·0	16[2]	12	0	1	13·8	(7·0–21·5)	18·6	(12·0–23·5)	17·4	(8·0–22·5)
IV	Neutral + *nil*	22	2	9·1	2[3]	1	0	0	25·5	(25·0–26·0)	26·0	(25·5–26·5)	26·3	(17·0–33·0)
V	Neutral + C	21	21	100·0	103[4]	68	8	19	10·1	(7·5–12·5)	14·5	(11·5–17·5)	—	
VI	Neutral + croton oil	25	2	8·0	2[3]	1	0	0	15·5	(14·0–17·0)	17·0	(16·0–18·0)	16·1	(12·0–23·0)
VII	Croton oil only	23	1	4·4	2[3]	1	0	1	21·0		22·5		16·4	(12·5–22·0)

1 = 2 tumours coalesced.
2 = 4 papillomata regressed.
3 = 1 papilloma regressed.
4 = many tumours coalesced as they grew; none regressed.

The first substance named in the treatment (column 2) of each group was the initiator, the second the promoter. The great majority of skin tumours were benign papillomata and the number attained its maximum before the end of treatment (column 6), being reduced thereafter either by complete regression or by coalescence into fewer larger tumours (column 7), a small number of which continued to develop to malignancy (i.e. the numbers in columns 8 and 9 are included in the numbers in column 7).

TABLE II.—*Tumour Production in Male and Female Mice by Fraction "C" from City Smoke, in Group V*

	Number of mice (1)	Total number of tumours: Maximum (2)	Total number of tumours: Final (3)	Number of tumours per mouse: Maximum (4)	Number of tumours per mouse: Maximum average (5)	Number of tumours per mouse: Final average (6)	Tumour incidence: Probably malignant: Number (7)	Probably malignant: Percent of total (8)	Malignant: Number (9)	Malignant: Percent of total (10)	Percentage which was malignant and probably malignant (11)
Males	15	79	55	15	5·3	3·7	2	3·6 (of 55)	16	29·1 (of 55)	32·7
Females	6	24	13	7	4·0	2·2	6	46·2 (of 13)	3	23·1 (of 13)	69·2

Group V.—The majority of the mice, otherwise healthy, were killed when their tumours became large and malignant or "probably malignant", but two had kidney disease and died before their tumours had grown to any size. Every mouse had multiple skin tumours. The tumour incidence in males and females is given in Table II. Although the males had on an average more tumours per mouse than the females, the difference was not significant either for the maximum average numbers (column 5) (d = 1·3, 2 × S.E. = 2·1) or for the final average numbers (column 6) after tumours had coalesced (d = 1·5, 2 × S.E. = 1·9). But when the proportions of tumours which became malignant or "probably malignant" were compared (column 11), the difference between the sexes was significant (d = 36·5, 2 × S.E. = 28·6). The average age of the females at tumour appearance was 10·8 months (range = 9·0–12·5 months) and that of the males was 9·8 months (7·5–11·5 months); the average age of the females at death was 14·2 months (12·0–17·0 months) and of the males was 14·5 months (11·5–17·5). Thus although the latent period was longer in the females, the tumours became malignant more rapidly than in the males, and a greater proportion of the tumours became malignant (and "probably malignant") in the females.

Group VI.—The only mice to produce skin tumours were 2 males, each having one small papilloma, one at the age of 14 months (on the forehead) and the other at 17 months (interscapular); the latter had disappeared at the time of death 2 months later. The mice in this group, although of tumour age, died sooner than those of other groups, most of them with diseased kidneys.

Group VII.—There was only one tumour mouse, a female with a skin papilloma on the left flank at the age of 21 months. That tumour disappeared before death 7 weeks later, but meanwhile another papilloma, which had appeared in the dorsal region a week or two after the first, had developed into a small epithelioma. The remaining mice died tumour-free at a similar age to those in Group VI and all but one had grossly diseased kidneys.

DISCUSSION

It is clear from these experiments that, under the conditions of treatment described, the neutral fraction of cigarette smoke can act as a definite tumour promoter. Some evidence has already appeared in the literature that whole tar from cigarette smoke may have tumour-promoting properties when applied after a known carcinogen such as 3 : 4-benzopyrene (Hamer and Woodhouse, 1956; Gwynn and Salaman, 1956); these workers found no proof of tumour-initiation. Gellhorn (1958) demonstrated convincingly the tumour-promoting activity of whole tobacco tar after the preliminary application of 3 : 4-benzopyrene; he found also that although croton oil promoted a higher incidence of carcinomata, tobacco tar increased the "conversion rate" of carcinomata from papillomata compared with croton oil. More recently Roe, Salaman and Cohen (1959) have shown that the phenolic fraction is a tumour promoter, the initiator in this case being 9,10-dimethyl-1,2-benzanthracene; they proved also that the neutral fraction is carcinogenic. Wynder and Hoffman (1960) mention the tumour-promoting properties of the phenol fraction and of the nicotine-free basic fraction.

As far as is known, the present series of experiments is the first in which the initiating substance has been, not one of the well-known carcinogens such as

3 : 4-benzopyrene, but a mixture (of proved carcinogenic power) obtained direct from city smoke. Although its components are still not wholly identified, fraction "C" was chosen deliberately in order to simulate more closely the actual conditions of daily life. That it is a strong carcinogen was shown once again by the mice of Group V, to which it was applied until death and in which the tumour incidence was 100 per cent; this result was the same as had been obtained in previous experiments with fraction "C" alone and any hypothetical promoting effect due to the preliminary treatment with neutral fraction would be quite obscured by the potency of fraction "C". That a minimal initiating dose was given to the mice in Group I was shown by the very low tumour incidence in that group.

The crucial results are given by Groups II and III; in these, fraction "C" applied in minimal dose as initiator was followed in Group II by neutral fraction and in Group III by croton oil as promoters. Croton oil is a well-known tumour promoter; it has also been shown to be a weak carcinogen, producing a number of papillomata which usually regress when treatment ceases, but Roe (1956) reported 7 malignant tumours in 20 mice which had been treated for from 55 to 72 weeks. In the present instance, applied alone throughout life (up to 21 months' treatment), it produced in Group VII only one malignant tumour and one papilloma which regressed in 23 mice living over one year.

In Group III 12 of the 24 mice developed a total of 16 skin tumours, of which 4 regressed and only one (which took 6 months to grow and was the only tumour of any size in that group) became malignant; the final tumour incidence was 33 per cent.

In Group II 12 of the 21 mice produced a total of 18 skin tumours, of which none regressed (but 2 coalesced as they increased in size) and 6 became malignant or probably malignant, 5 of them attaining quite a large size in from 2 to 4 months. Thus although the latent period (see Table I) was much longer with neutral fraction as promoter, there was a much shorter time between tumour appearance and death than with croton oil as promoter. While the differences in tumour incidence are not statistically significant, a comparison of the charts of tumour growth was most convincing and showed that under the conditions of the experiment a 10 per cent solution of neutral fraction applied three times a week was a more powerful tumour promoter than a 0·5 per cent solution of croton oil applied twice weekly.

The present experiments provide less certain evidence of tumour intiation by neutral fraction; with what was intended to be a minimal dose (Group IV) papillomata appeared in 2 mice out of 22, but one regressed and the other was far from the site of painting. When, in Group VI, after this preliminary treatment with neutral fraction, croton oil was applied as in Group II as a promoter, again only 2 mice developed skin papillomata, no more than might be expected from the use of croton oil alone. While this might mean that the original dose was too small to give tumours, in previous experiments in which a 10 per cent solution of neutral fraction was applied throughout life to mice of the A and C57BL strains no skin tumours were observed. On the other hand Roe, Salaman and Cohen (1959) obtained proof of complete carcinogenesis with neutral fraction when they produced papillomata and two skin carcinomata in 5 mice living up to 60 weeks (from 37 survivors at 30 weeks), painting being stopped after 47 weeks; they applied neutral fraction three times weekly at a dose of 40 mg. at each painting.

In the present work the dose of neutral fraction, forming about 18 per cent by weight of the whole original tar (Clemo, 1958), was approximately 6·4 mg. per mouse at each painting. The neutral fraction used by Roe, Salaman and Cohen (1959) which apparently contained the ester fraction removed from our neutral fraction, formed approximately 56 per cent by weight of the original tar. It is thus not possible to compare strictly the individual doses used by ourselves and by Roe *et al.* (1959). Gellhorn (1958) gave approximately 50 to 60 mg. of tar per week to each mouse ; our dose of 19·2 mg. neutral fraction per week probably represents about twice as much neutral fraction as would be present in his dose. Although Gellhorn used the same strength of croton oil, he was giving three times as much per week as in our experiments ; he obtained far more tumours with croton oil than he did with the tar (as promoters), and given the differences in dosage the present results are not inconsistent with his.

It was noticed in the present experiments that mice receiving croton oil throughout their lives (Groups III, VI and VII) died much earlier than those in other groups (except Group V where they all developed tumours) and the majority had severe kidney disease.

SUMMARY

Experiments are described in which a definite tumour-promoting effect was observed when the neutral fraction from cigarette smoke was applied to C57BL mice after an initiating dose of fraction " C " from city smoke. Eighteen skin tumours, of which 6 became malignant or " probably malignant " were produced in 12 out of 21 mice painted in the interscapular region throughout their lives (up to 28 months). Similar treatment with croton oil after fraction " C " resulted in 16 skin tumours, of which 4 regressed and one became malignant, in 12 out of 24 mice.

There was little evidence of either a tumour-initiating or a complete carcinogenic effect with a small dose of neutral fraction. No more tumours were produced with croton oil applied after neutral fraction than were produced by croton oil alone.

When fraction " C " was applied throughout life, the latent period was longer in females than in males, but tumour growth was more rapid in the females and a significantly greater percentage of tumours in females developed to malignancy.

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REFERENCES

CLEMO, G. R.—(1958) *Tetrahedron*, **3**, 168.
Idem AND MILLER, E. W.—(1957) *Brit. J. Cancer*, **11**, 403.
Iidem AND PYBUS, F. C.—(1955) *Ibid.*, **9**, 137.
GELLHORN, A.—(1958) *Cancer Res.*, **18**, 510.
GWYNN, R. H. AND SALAMAN, M. H.—(1956) *Rep. Brit. Emp. Cancer Campgn.*, **34**, 193.
HAMER, D. AND WOODHOUSE, D. L.—(1956) *Brit. J. Cancer*, **10**, 193.
KOTIN, P.—(1956) *Cancer Res.*, **16**, 375.
ROE, F. J. C.—(1956) *Brit. J. Cancer*, **10**, 72.
Idem, SALAMAN, M. H. AND COHEN, J.—(1959) *Ibid.*, **13**, 623.
WYNDER, E. L. AND HOFFMAN, D.—(1960) *Proc. Amer. Ass. Cancer Res.*, **3**, 164.

THE CHEMICAL INDUCTION OF BREAST TUMOURS IN THE RAT: HORMONAL FACTORS IN TUMOUR PRODUCTION

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MAMMARY tumours in the rat can be induced by a variety of methods. These include intensive treatment with hormone preparations, e.g. growth hormone (Moon *et al.*, 1950) and oestrogens (Geschickter, 1939; Mackenzie, 1955), by the administration of aminofluorene compounds (Bielschowsky, 1944, 1947; Symeonidis, 1954) and by certain carcinogenic hydrocarbons. These latter substances have been administered by various routes, but the most rapid method of induction has been reported by Huggins, Briziarelli and Sutton (1959) giving methylcholanthrene daily by stomach tube, a technique originally described by Shay *et al.* (1949).

Painting the skin of the rat with an oily solution of 9 : 10-dimethyl-1 : 2-benzanthracene (DMB) at fortnightly intervals is a highly effective method of inducing breast tumours. In initial experiments (Howell, 1959) it was shown that 77 per cent of female rats developed mammary tumours in an average time of 4·75 months, and furthermore, a single application of the carcinogen gave a tumour incidence of 75 per cent, although in this instance the average induction time was extended to 12 months. The present paper is concerned with the effects of gonadectomy, hormone supplements and the effects of normal and pathological lactation on tumour development following skin application of DMB. The experiments on lactation were undertaken since Marchant (1958) has shown that breast tumour development in IF mice is inhibited by full lactation, and that unilateral removal of the nipples in lactating IF mice allows the development of breast tumours on the side without nipples, but not on the normal side (Marchant, 1959).

MATERIALS AND METHODS

General

The animals were derived from two sources; entirely outbred laboratory stock, and from the Birmingham strain (Laboratory Animals Bureau Catalogue of Uniform Strains, No. 626, 1953).

In all experiments, the animals whose age at the start of carcinogenic treatment varied between 2 and 4 months were kept in galvanized wire-mesh cages, never more than 5 rats to a cage, and were given rat cube (Heygate & Sons, known as the Thompson diet) and water *ad lib.* Twenty drops of a 1·6 per cent solution of DMB in olive oil was applied to the skin at fortnightly intervals, 5 drops to each side of the ventral and dorsal surfaces; a single treatment averaged 1·3 ml. or 20·8 mg. DMB. After the first month of treatment all the animals were examined at fortnightly intervals to determine the presence of palpable breast tumours. When a tumour was found, treatment was stopped and usually the animal was killed,

although sometimes the tumours were allowed to grow. In animals not developing breast tumours, treatment was continued until death, or until excoriation of the skin, sometimes with the development of skin tumours necessitated stopping treatment.

Experimental groups

Experiment A.—In this experiment a total of 35 laboratory stock males and 38 Birmingham strain female rats divided into 5 groups were used ; the appropriate treatment of each group is detailed in Table I. Castration or ovariectomy was

TABLE I (*Experiment A*).—*Experimental Groups and Results*

Group	Treatment	Number of Rats F	Number of Rats M	Rats "at risk" F	Rats "at risk" M	Breast tumours F	Breast tumours M	Average induction time (months) F	Average induction time (months) M	Average number paintings F	Average number paintings M	Average time to death (months) F	Average time to death (months) M
1	DMB only	22*	8	22	8	17	0	4·75	—	11	18	5·3	9·3
2	Gonadectomy + DMB	9	9	9	9	0	0	—	—	16	17	8	8·8
3	Hexoestrol + DMB	10	9	7	5	2	0	7·2	—	14	15	7·2	7·5
4	Progesterone + DMB	10	—	6	—	3	—	7·8	—	18	—	9	—
5	Hexoestrol + Progesterone + DMB	9	9	9	9	5	1	7	9	14	15	7	7·5

* = Animals of previous experiments
Hexoestrol = 7 mg. pellets
Progesterone = 5 mg. intra-peritoneal injection every 14 days.
All groups received DMB every 14 days.

performed when the animals were 6 weeks old, and treatment with the carcinogen was commenced 4 weeks after the operation. The hexoestrol pellets, 7 mg. each (Boots Pure Drug Co.), were inserted at 4 weeks of age by trochar and cannula into the subcutaneous tissue between the scapulae ; DMB painting was started 4 weeks following the implant. The animals in these groups surviving 4 months or more received a second 7 mg. implant. Progesterone, given to some animals, was administered by intra-peritoneal injection, in a dose of 5 mg. dissolved in olive oil at fortnightly intervals, alternating with the application of DMB.

TABLE II (*Experiment B*).—*Experimental Groups and Results*

Group	Treatment	Number of Rats	Number "at risk"	Breast tumours	Average induction time (months)	Average number of litters	Average number of paintings	Average time to death (months)
6	Lactation + DMB	12	11	1	6	3	17	8·8
7	Lactation + Unilateral nipple removal + DMB	12	10	3	8·8	3	15	7·5

Experiment B.—In this experiment (Table II) 24 laboratory stock females were used ; they were divided into two equal groups, and the animals were kept in individual cages. One group of 12 rats were mated, and after each had produced and suckled a litter, treatment with the carcinogen was started. The second

group was treated identically except that all the nipples on the left side of the body were excised following suckling of the first litter, so that the breasts on that side could not be suckled thereafter. Throughout both these experiments, breeding followed by lactation and suckling combined with carcinogen treatment was continued until the death of the animals or until the state of the skin necessitated killing them.

Post mortem and histological methods

Blocks of tissue from all the breast tumours were preserved for microscopic study. When possible blocks of tissue from the right inguinal breast in animals with and without breast tumours were preserved for purposes of comparison ; tissue was also preserved from all organs showing gross pathological changes.

All tissues were fixed in 4 per cent formaldehyde-saline. Sections were stained with Ehrlich's haematoxylin and eosin, Weigert's haematoxylin and Van Gieson and by Lawson's elastic stain. On occasion frozen sections were cut and stained for fat.

RESULTS

The results of Experiments A and B are detailed in Tables I and II.

Experiment A (Table I)

Group 1.—Eight males survived for an average period of 9·3 months, receiving an average of 18 paintings each. None developed breast tumours although skin tumours were present in 7. One had a squamous cell carcinoma of the ear duct.

The results for normal females have been abstracted from previous experiments already reported (Howell, 1959). Suffice it to state here that 22 females were treated with the carcinogen and 17 developed breast tumours in an average time of 4·75 months.

Group 2.—The 9 castrated males survived for an average period of 8·8 months, receiving an average of 17 paintings ; 5 developed skin tumours and 2 developed squamous carcinomata arising from ear ducts, but no breast tumours were found.

Nine ovariectomized females survived for an average period of 8 months, receiving an average of 16 paintings but despite this no animal developed a breast tumour. Seven developed skin tumours and 4 had squamous carcinomata arising from ear ducts.

Group 3.—Survival of the male animals in this group was poor ; 4 died after only 2 or 3 paintings. The remaining 5 survived for an average period of 7·5 months, receiving an average of 15 paintings, but none of them developed breast tumours. Two had skin tumours.

Survival in the females was better, but 3 animals were lost due to cannibalism after treatment had been in progress for 6 months, none of which had had palpable breast tumours before death. The remaining 7 were treated for 7·2 months receiving an average of 14 paintings. Two developed breast tumours ; one was found after 5·5 months, and the other in the sole survivor of the group, was found at 9 months. None had skin tumours.

Group 4.—Only females were included in this group. Three of the 10 died very early in the experiment and one was lost due to cannibalism, the remaining 6 were treated for an average period of 9 months receiving an average of 18

paintings. Two developed breast tumours at 6·25 months and a further animal developed a breast tumour after 11 months, this animal was the sole survivor. None had skin tumours.

Group 5.—Nine males survived for an average period of 7·5 months receiving an average of 15 paintings. One animal developed a breast tumour at 9 months. Two other animals had skin tumours, and 2 had squamous cell carcinoma of the ear ducts.

Nine females survived for 7 months and of these, 5 developed breast tumours, the first appearing after 3·5 months and the last one after 10·5 months. The average tumour induction time was 7 months. Two rats had skin tumours, and one had a squamous cell carcinoma of the ear duct.

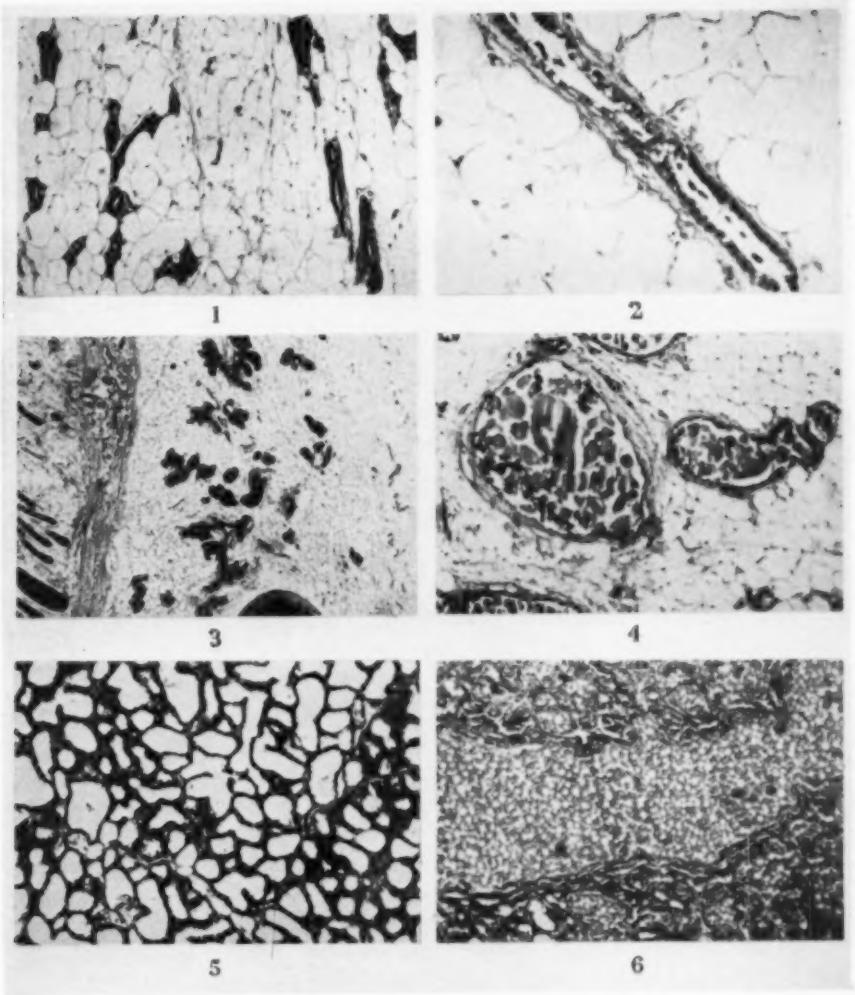
Experiment B (Table II)

Group 6.—Eleven rats survived for an average period of 8·8 months, during which time they received an average of 17 paintings and produced and suckled an average of 3 litters per rat ; one rat had 5 litters, 5 had 4 litters, one had 3 litters, one had 2 litters and 3 had a single litter. One rat died early in the experiment due to sepsis in the genital tract. One breast tumour was found in the rat that had had 2 litters ; this was of an unusual appearance on gross examination and was subsequently shown to be a simple fibro-adenoma. Seven rats developed skin tumours and 4 had squamous carcinomata of the ear ducts.

Group 7.—Ten rats survived for an average period of 7·5 months. In this time they received an average of 15 paintings and produced and suckled an average of 3 litters per rat; two rats had 5 litters, one had 4 litters, 4 had 3 litters, 2 had 2

EXPLANATION OF PLATES

FIG. 1.—Normal female rat breast. H. and E. ×37.
FIG. 2.—Duct in normal female rat breast showing elastic fibres in the wall. Elastic ×73.
FIG. 3.—Normal male rat breast showing solid acini. H. and E. ×18.
FIG. 4.—Dilatation of ducts filled with globules of secretion. Female with hexoestrol implant. H. and E. × 75.
FIG. 5.—Lactating DMB-treated breast. H. and E. ×73.
FIG. 6.—Lactating DMB-treated breast. To show cystic dilatation of a duct on the side from which the nipples had been removed. H. and VG. ×73.
FIG. 7.—Poorly differentiated carcinoma of breast. H. and E. ×75.
FIG. 8.—Duct-like structures in carcinoma of breast. H. and E. ×87.
FIG. 9.—Intracystic papilliferous growth. H. and E. ×87.
FIG. 10.—Trabecular growth showing resemblance to human scirrhous carcinoma. H. and E. ×113.
FIG. 11.—From an area of alveolar-like growth showing secretory changes in cells. H. and E. ×187.
FIG. 12.—Invasion of muscle. H. and VG. ×87.
FIG. 13.—Fragmentation of elastic fibres round ducts. Elastic ×87.
FIG. 14.—Fragmentation of elastic fibres around a duct. The elastic tissue appears as distinct blobs in the wall of the duct. Elastic ×335.
FIG. 15.—Early proliferation of cells lining a ductule. H. and E. ×187.
FIG. 16.—Marked cellular proliferation within a ductule. H. and VG. ×176.
FIG. 17.—Intraduct carcinoma. H. and E. ×75.
FIG. 18.—Intraduct carcinoma with early infiltration. Note disruption of elastic fibres at point of infiltration. Elastic ×113.
FIG. 19.—Squamous cell carcinoma of ear duct. H. and E. ×95.
FIG. 20.—Fibrosarcoma. Note dermal appendages included in the tumour. H. and VG. ×18.
FIG. 21.—Hair follicles within a fibrosarcoma. H. and VG. ×140.
FIG. 22.—Cystic dilatation of Zymbal's gland associated with squamous metaplasia. H. and E. ×32.



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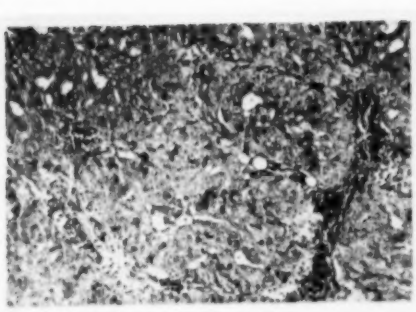

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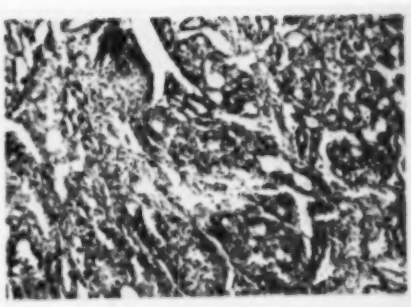

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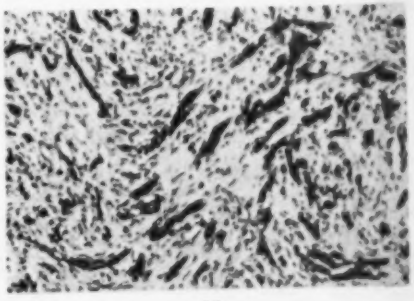

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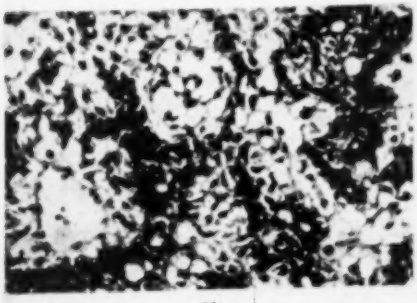

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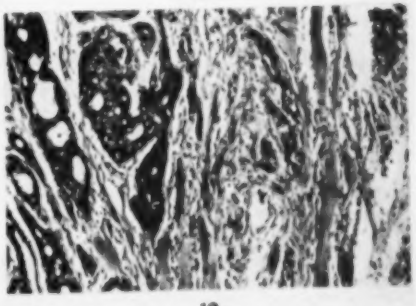

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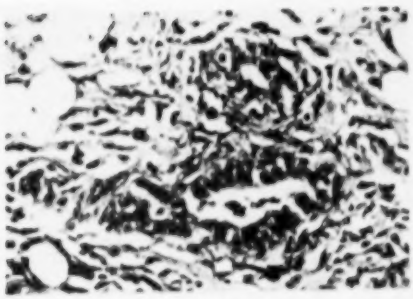

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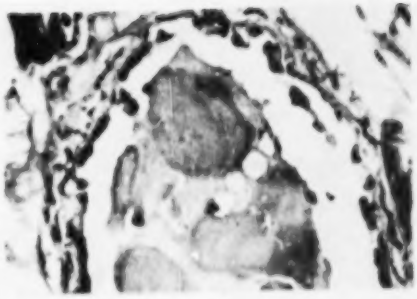

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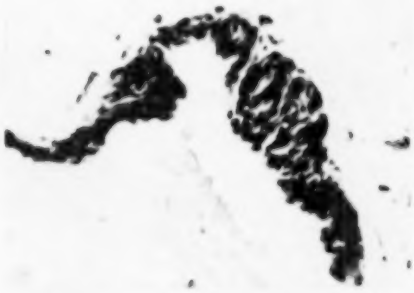

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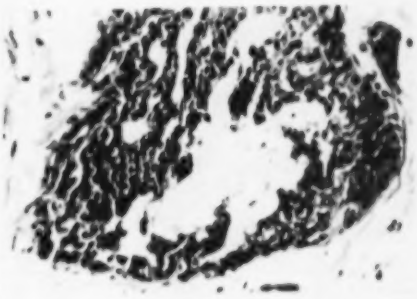

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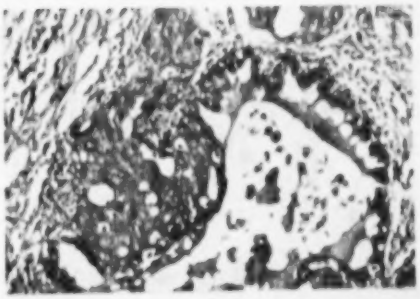

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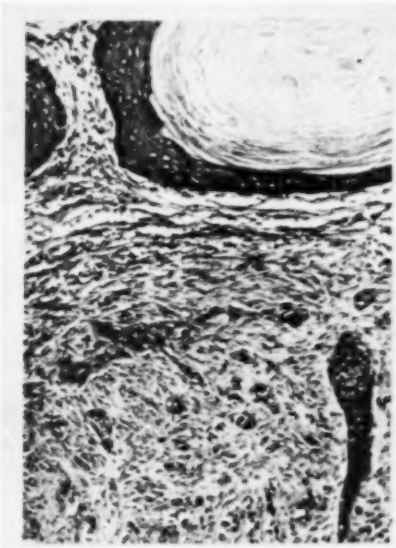

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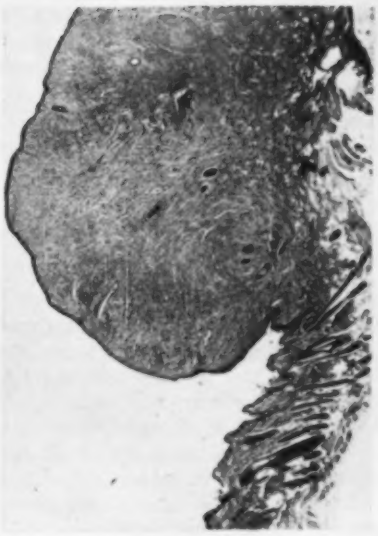

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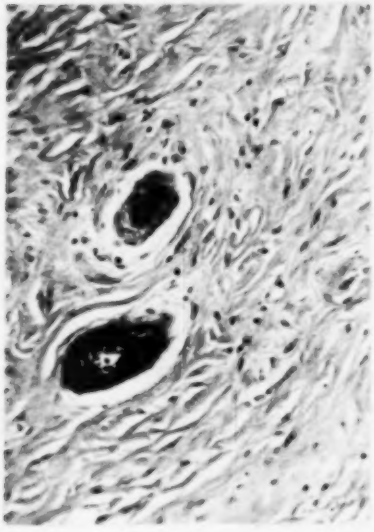

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litters and one had a single litter. Two rats had died early as a result of sepsis in the genital tract. Three animals developed breast tumours ; one of these, found at 11 months, was on the side with intact nipples in a rat that had had 5 litters. The other 2 animals developed breast tumours at 7·75 months on the side from which the nipples had been removed. One of these rats had 3 separate breast tumours and had had 3 litters ; the other rat with a single breast tumour had had 4 litters. Two rats had skin tumours, and one had a squamous carcinoma of the ear duct.

Pathology

Normal female and male rat breast

The adult virgin female rat breast has well developed ducts within the lumen of which is frequently rather dense eosinophilic secretion. The ducts are lined internally by a single layer of cuboidal cells, external to which is a layer of myo-epithelium surrounded by a fibrous mantle which decreases in thickness as the ducts branch and become smaller (Fig. 1). Elastic fibres are present within the fibrous tissue encircling the ducts, and by definition a duct must have elastic fibres in its wall (Fig. 2). Arising from the ducts are small bud-like projections forming acini frequently with ill-defined lumina. The acinar cells, and frequently the cells lining the ducts, may contain secretory vacuoles.

The male rat breast compared with other male rodents shows an unusual degree of development throughout adult life. There are fewer ducts as compared with the female, but solid acini are more numerous, the acinar cells being large and eosinophilic (Fig. 3). Both ducts and acini contain eosinophilic secretion, but no evidence of secretory activity was observed in the lining cells.

Histology of breasts of DMB-treated animals without tumours

Ovariectomy made comparatively little difference to the histological structure of the breast except that duct and acinar development was slightly less than in the intact rat. The cells forming the acini were larger and the cytoplasm was more eosinophilic than in the intact rat and they showed no evidence of secretory activity. Castration was also without marked effect on the histological structure of the breast except that in some animals acinar development was less than in the intact male.

Females with hexoestrol implants showed great proliferation and extension of the duct systems, which were also greatly dilated and filled with multiple globules of dense, eosinophilic, sometimes basophilic secretion (Fig. 4). The cells lining many of the ducts showed marked secretory activity. The fibrous stroma of the breast was increased in amount, replacing the normal adipose tissue and the fibrous mantle around the ducts was thicker and rather acellular. Chronic inflammatory cells were present in the stroma. Initially there was also some proliferation of acini, but this was later overshadowed by the duct development. The breast tissues of male animals with hexoestrol implants were similar in appearance to those of the female.

The combination of hexoestrol implants wtih progesterone injections gave the same histological picture in both females and males as with hexoestrol alone, progesterone apparently made no difference to breast histology. The females

receiving progesterone injections without hexoestrol showed similar histological appearances to the females without hormonal supplement.

The females that were allowed to breed and lactate during carcinogen treatment showed changes in the breasts dependent upon the stage of pregnancy or lactation at which they were killed or died and no abnormalities of the breast tissues were observed (Fig. 5).

In the rats that had had all the nipples removed from one side of the body, the contralateral breast showed normal pregnancy or lactational changes. On the side with the nipples removed, the breast tissues showed changes of pregnancy or lactation but these were not so well developed as those on the normal side and in some of these animals there was very considerable cystic dilatation of ducts which were filled with globules of dense secretion (Fig. 6).

Breast tumours

The breast tumours were first identified as small, discrete, freely mobile nodules in the subcutaneous tissues. They tended to grow fairly rapidly and, if the animal was not killed, they attained a considerable size. They could usually be dissected from the surrounding tissues with ease ; fixation to the deeper tissues was uncommon, but fixation to skin, sometimes associated with ulceration occurred frequently, especially in the larger tumours.

On gross inspection, the tumours had a smooth, sometimes lobulated surface. They were usually yellowish-pink in colour with a soft fleshy consistency, and the reshly cut surface frequently exuded a small quantity of a pale milky fluid. In the larger tumours areas of cystic degeneration with and without haemorrhagic necrosis were common, sometimes with superimposed infective changes.

On histological examination, the breast tumours with a few exceptions were adenocarcinomata, with a complex histological structure which varied not only between tumours but also between different parts of the same tumour.

The tumours showed all degrees of differentiation ; frequently they consisted of solid sheets of cells with scanty stroma and no evidence of differentiation (Fig. 7). Usually however they showed closely applied duct-like structures with a cylindromatous appearance, the intimate structure of which had a solid or cribriform pattern (Fig. 8). Sometimes cysts were present associated with intracystic papillary growth (Fig. 9). Not infrequently areas of solid acinar and trabecular growth were present ; these were usually situated at the periphery of the tumours and the appearances were somewhat similar to the human scirrhous breast carcinoma (Fig. 10). The stroma in these tumours was fairly abundant, consisting of cellular fibrous strands closely investing the epithelial elements. Sometimes, thicker and more hyaline fibrous trabeculae transected the tumour giving rise to a distinctly lobular appearance.

In other types of tumour, alveolar-like growth was observed in which the intimate structure showed a resemblance to the lactating breast, with secretory vacuoles in the epithelial cells lining the alveoli (Fig. 11). The stroma in this type of growth was scanty and consisted of thin fibrous strands separating alveoli from each other, although sometimes coarser bands were also present breaking the tumour into lobules.

Unlike the chemically induced mouse breast tumours squamous metaplasia was rare ; when present it was always adjacent to an area of necrosis or other inflam-

matory reaction. Metastases in lymph glands or other organs were never observed but invasion of muscle was frequently observed (Fig. 12).

Changes in breast tissue antecedent to tumour formation were rather ill defined. Generally after DMB treatment the fibrous mantle around ducts increased slightly in amount and became thicker. In some instances this was associated with fragmentation and condensation of the elastic fibres around the ducts, so that the elastic tissue appeared as distinct blobs (Fig. 13 and 14). In some ducts there was proliferation of the lining cells, so that these became 2–3 cells thick and almost filled the lumen (Fig. 15 and 16), in certain instances this change progressed to frank intraduct carcinoma sometimes associated with invasion of the duct wall and early infiltration of the stroma (Fig. 17 and 18). Another change consisted of the development of acini which frequently had vacuoles in the lining cells ; these appeared to bud out from ducts and appeared more marked than acinar development in the normal virgin rat breast.

Other pathological changes

In addition to breast tumours, certain other neoplastic lesions were found in these rats. The commonest, found in rats of most groups, were skin tumours, which histologically were mainly kerato-acanthomata. They tended to develop later than the breast tumours and were most numerous in animals without breast tumours. In 15 rats, 5 male and 10 female, squamous cell carcinomata arising in the ear ducts were found (Fig. 19). These produced large swellings adjacent to the ears and at post-mortem examination were nearly always secondarily infected. In one of these animals there were massive deposits of secondary squamous cell carcinoma in both lungs, which at first, on gross inspection, was confused with bronchiectasis, but histology showed undoubted carcinoma.

In 12 rats there were lymphomatous lesions, characterized by pallor and enlargement of lymph nodes, thymus, liver and spleen. Histology showed destruction of the normal architecture of these organs with replacement and infiltration by primitive cells, the appearances of which were suggestive of white cell precursors.

In 6 rats, 4 males and 2 females, interesting fibrosarcomatous tumours were found (Fig. 20), consisting of interlacing whorling bundles of collagen which varied considerably in cellularity. Some were hyaline and relatively acellular, but others were extremely cellular, with large, plump fibroblast-like cells showing occasional mitotic figures. Within the tumours there were sometimes occasional epithelial remnants suggestive of breast ducts which appeared to be incorporated by infiltrative and expansive growth of the tumour. Since hair follicles and other skin appendages were sometimes also incorporated (Fig. 21), their presence suggests the tumours arose in tissues more superficial than those of the breast, and from study of early examples of these tumours, they appeared to arise in the dermis immediately subjacent to the epidermis.

Most of the rats, both male and female, receiving hexoestrol implants had enlarged pituitary glands. Histologically these all showed proliferation of basophil cells. No enlargement of the pituitary was observed in rats treated with DMB only.

DISCUSSION

It is difficult to compare directly the results of these experiments with other reported experiments on the chemical induction of rat breast tumours since the

carcinogen and/or its mode of administration differ, as do the various hormone preparations used.

Shay, Harris and Gruenstein (1952) and Shay, Gruenstein and Harris (1956) administered 2 mg. of methylcholanthrene daily by stomach tube to normal female rats and 89 per cent developed breast tumours in an average time of 6·5 months. The age of the animal at the start of treatment had some bearing on tumour yield and induction time, since using older animals, the tumour incidence fell, and the average induction time increased. Employing the same technique but with rats of 42 days old and increasing the daily dose of methylcholanthrene to 10 mg., Huggins, Briziarelli and Sutton (1959) obtained breast tumours in every animal treated in a mean time of 55·9 days. This represents one of the most rapid methods of obtaining the tumours. These results can be compared with those obtained by applying DMB to the skin ; with a 0·5 per cent solution of the carcinogen, 75 per cent developed breast tumours in an average time of 7·25 months, whereas using a 1·6 per cent solution, the average induction time fell to 4·75 months although the tumour incidence was unaltered (Howell, 1959). Geyer *et al.* (1953) using intravenous DMB also showed that the quantity of carcinogen administered influenced the yield and induction time of tumours.

In the present experiments DMB-treated normal male rats did not develop breast tumours. Geyer *et al.* (1951) also failed to obtain breast tumours in males following intravenous DMB, as did Dao and Sunderland (1959), and Kim and Furth (1960) giving intragastric methylcholanthrene. Shay *et al.* (1952) found that 43 per cent of normal males developed breast tumours in 12·8 months, but only one was a typical adenocarcinoma, the remainder being spindle cell or collagenous tumours, and in one instance a fibro-adenoma. In 3 intact males in the present experiments fibrosarcomatous tumours were found, but these are not considered to be breast tumours within the context of this paper.

Ovariectomy followed by DMB completely inhibited breast tumour development despite continuation of treatment for an average period of 8 months. Shay *et al.* (1952) obtained breast tumours in 37 per cent of ovariectomized females, but the average induction time was 12·8 months, rising to 15·5 months in older animals. Huggins, Briziarelli and Sutton (1959) also observed a reduction in tumour incidence following ovariectomy.

No breast tumours were produced in males that were castrated then given DMB. No details have been found in the literature concerning the effect of castration on breast tumour production following administration of carcinogenic hydrocarbons except for Dao and Sunderland (1959) who obtained tumours in 2 castrated animals.

When intact females were treated with hexoestrol implants concurrently with the carcinogen only 2 out of 7 rats developed tumours. Although these observations are based on a small number of animals they tend to support Shay *et al.* (1956) who found that oestrogen caused a sharp fall in tumour incidence and prolongation of average induction time and they made the interesting suggestion that the fall in tumour incidence might be due to development of oestrogen induced pituitary tumours with consequent "physiological hypophysectomy", thus rendering the animals insensitive to the carcinogen. In this connection it is significant that hypophysectomy completely inhibited breast tumour development following intramuscular injections of DMB (Noble and Walters, 1954) and also following intragastric methylcholanthrene (Huggins, Grand and Brillantes, 1959).

Intact DMB-treated males with hexoestrol implants did not develop breast tumours, but Shay *et al.* (1956) obtained them in 91 per cent of oestrogenized, methylcholanthrene-treated males after 7·5 months ; castration before oestrogen implantation made no significant difference to the tumour incidence.

It should be mentioned that in the present experiments a small group of male and female rats received hexoestrol implants in exactly the same way as those given the carcinogen, but no breast tumours were observed over a period of 18 months.

Progesterone injections plus DMB were given to female rats only, and 3 out of 6 animals developed tumours in 7·8 months, however the dose of progesterone used was substantially less than in other experiments reported in the literature. Shay *et al.* (1952) with a 50 mg. progesterone implant observed a moderate fall in tumour incidence with a slightly prolonged average induction time. However, Huggins, Grand and Brillantes (1959) giving 4 mg. of progesterone daily, obtained breast tumours in all the animals treated with a reduction in average induction time which was even greater using the synthetic preparation 9–2 Bromo-11-ketoprogesterone.

The combination of hexoestrol, progesterone and DMB gave breast tumours in 5 out of 9 female rats after an average induction time of 7 months. Unlike the groups given the hormones separately, the incidence of breast tumours approached that observed in rats treated with DMB alone, although the induction time was 2·25 months longer. Scholler and Carnes (1958) found no effect on tumour production when intravenous DMB was given with oestrogen and progesterone.

Nine male rats were treated for an average period of 7·5 months with the combined hormones and DMB ; one animal, the sole survivor, developed a breast tumour after 9 months. This suggests that by suitable hormonal preparation breast tumours can be induced in the male. In this respect it has been shown that the relative proportion of oestrogen to progesterone is of crucial importance in the development of the normal breast (Folley, 1952) and hence the proportions of the hormones given to animals in carcinogenesis experiments may be of similar significance.

It is commonly accepted that the incidence of carcinoma of the breast in the human female is reduced by breast feeding, and experimentally, Marchant (1958) has shown that breeding with lactation reduces the incidence of chemically induced breast tumours in IF mice. In the present experiments, breeding, lactation, and DMB treatment were continued for an average of 8·8 months but only one of 11 rats developed a tumour, found at 6 months ; this was a fibro-adenoma in contrast to the adenocarcinoma usually induced. Dao and Greiner (1960) using intragastric methylcholanthrene also observed a marked fall in tumour incidence when rats were allowed to breed and suckle their young.

Breast tumour development following skin application of DMB is probably due to a systemic effect caused by absorption of the compound, since it is equally effective in producing breast tumours when given by intranasal instillation (Howell, unpublished observations). One of the factors inhibiting breast tumour development in lactating animals may be that the carcinogen is excreted in the milk leaving insufficient time for it to act on the breast tissues. Shay *et al.* (1950) have shown that methylcholanthrene is excreted in the milk, and in the present experiments milk expressed from lactating breasts of DMB-treated animals fluoresced in ultraviolet light.

In Group 7 the nipples were removed from one side of the body to determine whether inhibition of tumour development in lactating rats was due to the hormonal

status and milk excretion, a technique described by Marchant (1959) using IF mice. The results however are inconclusive since only 3 animals developed tumours. Two tumours, found at 7·25 months, were on the side from which the nipples had been excised; the third rat had a tumour on the normal side but this did not develop until 10 months. It may also be significant that one of the animals with tumours on the side without nipples developed 3 distinct tumours, all found at the same time.

The ear duct tumours observed in 15 animals were all squamous cell carcinomata apparently arising in Zymbal's gland, a sebaceous gland situated near the tympanic membrane. Cystic dilatation of the gland associated with squamous metaplasia of the lining epithelium (Fig. 22) preceded tumour development. Similar tumours have been produced by 2-acetylaminofluorene (Bielschowsky 1944, and Skoryna, Ross and Rudis, 1951) and intranasal instillation of DMB (Howell, unpublished observations). Since the breast is a modified sebaceous gland, it is perhaps not surprising that tumours should also arise in Zymbal's gland, although these tumours have not been reported following intragastric methylcholanthrene.

SUMMARY

1. Experiments are described which show that painting the skin of female rats with an oily solution of DMB is an effective method of inducing breast tumours. It has been shown that hormonal factors are important for tumour development; ovariectomy, continued breeding and lactation prevent their development and there is a suggestion, based on a small number of animals, that hexoestrol implants with and without progesterone injections reduce the tumour incidence and prolong the induction time.

2. Experiments have shown that male rats do not develop breast tumours, neither do castrated males. Hexoestrol implants were without effect on breast tumour development, but one out of nine male rats given both hexoestrol and progesterone developed a single breast tumour.

3. Certain other neoplastic changes in rats treated with DMB are briefly described.

4. The results of these experiments are compared with other results on the induction of rat breast tumours reported in the literature.

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REFERENCES

Bielschowsky, F.—(1944) *Brit. J. exp. Path.*, **25**, 1.—(1947) *Brit. med. Bull.*, **4**, 382.
Dao, T. L. and Greiner, M.—(1960) *Proc. Amer. Ass. Cancer Res.*, **3**, 103.
Idem and Sunderland, H.—(1959) *J. nat. Cancer Inst.*, **23**, 567.
Folley, S. J.—(1952) In 'Marshall's Physiology of Reproduction'. Ed. Marshall, F. H. A. London (Longmans), p. 546.
Geschickter, C. F.—(1939) *Science*, **89**, 35.

GEYER, R. P., BLEISCH, V. R., BRYANT, J. E., ROBBINS, A. N., SASLAW, J. M., AND STARE, F. J.—(1951) *Cancer Res.*, **11**, 474.
Idem, BRYANT, J. E., BLEISCH, V. R., PEIRCE, E. M., AND STARE, F. J.—(1953) *Ibid.*, **13**, 503.
HOWELL, J. S.—(1959) *Acta Un. int. Cancr.*, **15**, 163.
HUGGINS, C., BRIZIARELLI, G., AND SUTTON, H.—(1959) *J. exp. Med.*, **109**, 25.
Idem, GRAND, L. C., AND BRILLANTES, F. P.—(1959) *Proc. nat. Acad. Sci., Wash.*, **45**, 1294.
KIM, U. AND FURTH, J.—(1960) *Proc. Amer. Ass. Cancer Res.*, **3**, 125.
MACKENZIE, I.—(1955) *Brit. J. Cancer*, **9**, 284.
MARCHANT, J.—(1958) *Ibid.*, **12**, 55.—(1959) *Nature, Lond.*, **183**, 629.
MOON, H. D., SIMPSON, M. E., LI, C. H. AND EVANS, H. M.—(1950) *Cancer Res.*, **10**, 549.
NOBLE, R. L. AND WALTERS, J. H.—(1954) *Proc. Amer. Ass. Cancer Res.*, **1**, 35.
SCHOLLER, J. AND CARNES, R. E.—(1958) *Ibid.*, **2**, 343.
SHAY, H., AEGERTER, E. A., GRUENSTEIN, M. AND KOMAROV, S. A.—(1949) *J. nat. Cancer Inst.*, **10**, 255.
Idem, FRIEDMANN, B., GRUENSTEIN, M. AND WEINHOUSE, S.—(1950) *Cancer Res.*, **10**, 797.
Idem, GRUENSTEIN, M., AND HARRIS, C.—(1956) *Acta Un. int. Cancr.*, **12**, 733.
Idem, HARRIS, C., AND GRUENSTEIN, M.—(1952) *J. nat. Cancer Inst.*, **13**, 307.
SKORYNA, S. C., ROSS, R. C., AND RUDIS, L. A.—(1951) *J. exp. Med.*, **94**, 1.
SYMEONIDIS, A.—(1954) *J. nat Cancer Inst.*, **15**, 539.

THE GRADUAL CONVERSION OF A SPONTANEOUS MOUSE MAMMARY CARCINOMA TO AN ASCITES TUMOUR

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THE capacity of tumours to convert from the solid to the ascitic type varies, spontaneous mammary adenocarcinomas in mice being regarded as amongst the most resistant in this respect (Klein and Klein, 1955). In the present experiment an attempt was made to convert 9 solid mouse mammary carcinomas, 5 spontaneous and 4 transplanted, to the ascitic form.

MATERIAL AND METHODS

Passage of solid tumour

The mammary tumours occurred in 3 strains each carrying the milk factor (Table I). When the tumours had attained a size of about 1·5 cm. in diameter they were removed and necrotic tissue was discarded ; the healthy tumour tissue was washed in physiological saline, minced with scissors and strained through fine metal-wire mesh by means of a small pestle under dropping salt solution.

TABLE I.—*Mammary Carcinomas used for Intraperitoneal Transfer in Order to Obtain Ascites Tumours*

Tumour No.	Type and generation	Histological type	Transfer generations attempted	Fate	Full name of strain
1	Spontaneous	Mucoid adeno-carcinoma (M)	33–37	Ascites tumour produced	Balb/C fostered on C3H/CB/Se
2	Transplanted (1)	Mucoid adeno-carcinoma (M)	23	Always solid abandoned	As above
3	Transplanted (1)	Polygonal cell carcinoma (B)	12	Mixed fluid & solid, transfer by fluid which failed to take at gen. 12	As above
4	Spontaneous	Mucoid adeno-carcinoma (M)	5	Always solid failed to take	C3H/CB/Se
5	Transplanted (3)	Polygonal cell carcinoma (B)	5	Always solid failed to take	As above
6	Spontaneous	Small spherical adenocarcinoma (A)	4	Always solid abandoned	Balb/C fostered on C3H/CB/Se
7	Spontaneous	Mucoid adeno-carcinoma (M)	3	Always solid abandoned	As above
8	Spontaneous	Small spherical adenocarcinoma (A)	1	No takes	RIII/Dm/Se
9	Transplanted (3)	Small spherical adenocarcinoma (A)	1	No takes	C3H/CB/Se

M = Mucoid adenocarcinoma of Olivi, Biancifiori and Barbieri (1955).
A = Type A of Dunn (1953).
B = Type B of Dunn (1953).
Figures in parentheses represent number of generations of transplantation.

The resulting tumour suspension was adjusted to contain about 50 per cent of tumour.

Cell counts on fluids

The total neoplastic and non-neoplastic cellular content of the peritoneal fluid was counted in a Burker's haemocytometer and a count of the relative numbers of each type of cell was made in smears stained by Wright's method. Thus an approximate estimation could be made of the percentage and actual numbers of neoplastic cells transferred.

Experimental procedure

The scheme of tumour transfer to successive generations was as follows: 1 ml. of the fluid extract of the tumour to be tested was injected intraperitoneally into 4 or 5 three-month old female mice of the strain of origin of the tumour. The hosts were allowed to survive as long as possible in order to obtain a maximum amount of solid tumour and peritoneal fluid for transfer to succeeding hosts. If solid tumour only resulted, this was suspended in the same way as the starting material and injected into the subsequent generation. If solid tumour and fluid was obtained, an extract of the solid part as well as the fluid were injected into the next generation. As soon as successful transfer of the fluid portion seemed to be established, transplantation of the solid portion ceased.

RESULTS

Development of the ascites tumour

Of the nine tested (Table I) only one spontaneous tumour converted to the ascites type. The others were abandoned: (*a*) because the extracts of the solid tumour failed to take; (*b*) because after many generations only solid tumour resulted (tumour 2) or (*c*) because, having obtained successful fluid transference for a few generations, finally no takes were obtained (tumour 3).

Fig. 1 shows the scheme of transfer to succeeding generations of tumour 1 (Table I). In the early generations both solid tumour and peritoneal fluid were transferred, but with the exception of the 5th, the fluid always failed to take. At the ninth generation and thereafter fluid took successfully, producing a diminishing amount of solid tumour until at the 33rd–37th generation, according to the line (Fig. 1), no solid tumour was obtained.

During the series of transfers of tumour No. 1, the solid fraction was usually modified by decrease in volume and the appearance of small pin-head nodules on the peritoneal surfaces. This was associated with increase in the amount and tumour-cell content of the fluid. But these features were not constant and where a bulky solid mass reappeared, the amount and tumour-cell content of the fluid usually decreased. Finally, the fluid fraction increased at the expense of the solid portion.

Maintenance of the ascites tumour

All the lines A–F have been maintained by fluid transfer until the present time, approximately 30 transfers having been performed. No solid tumour was observed and the neoplastic cell content of the fluid varied between 85 and 95 per cent.

Tumour-cell content of the peritoneal fluid

A relationship between the percentage of tumour cells in the peritoneal exudate and the mean survival time of the hosts was sought (Fig. 1). During the period of conversion from solid to ascites tumour, i.e. from the start until the 30th

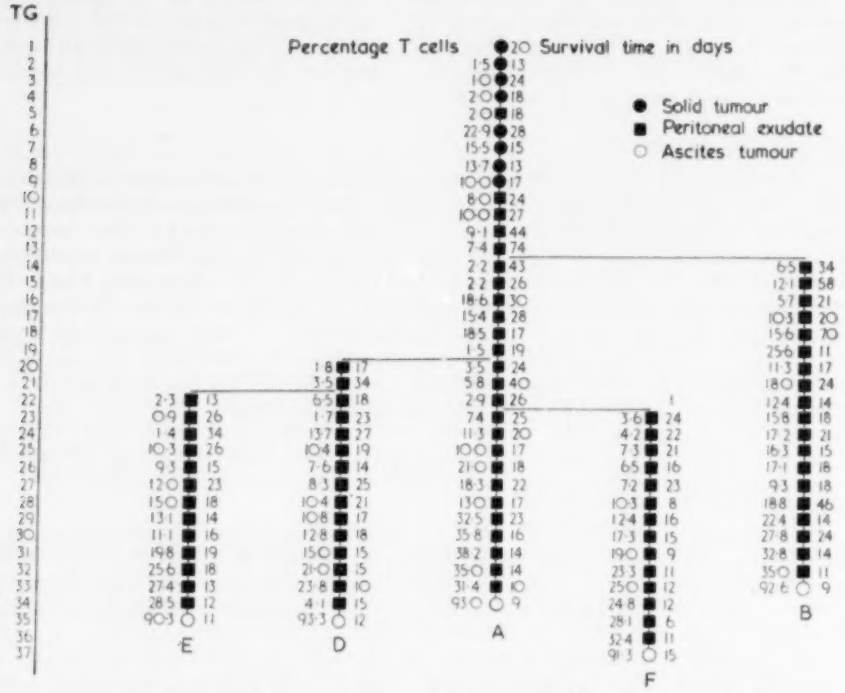


FIG. 1.—Scheme of transfer of solid tumour No. 1 or peritoneal fluid to succeeding generations of hosts and point of conversion to ascites tumour.

TG = transfer generation
A–F = main line and sublines of transfer.

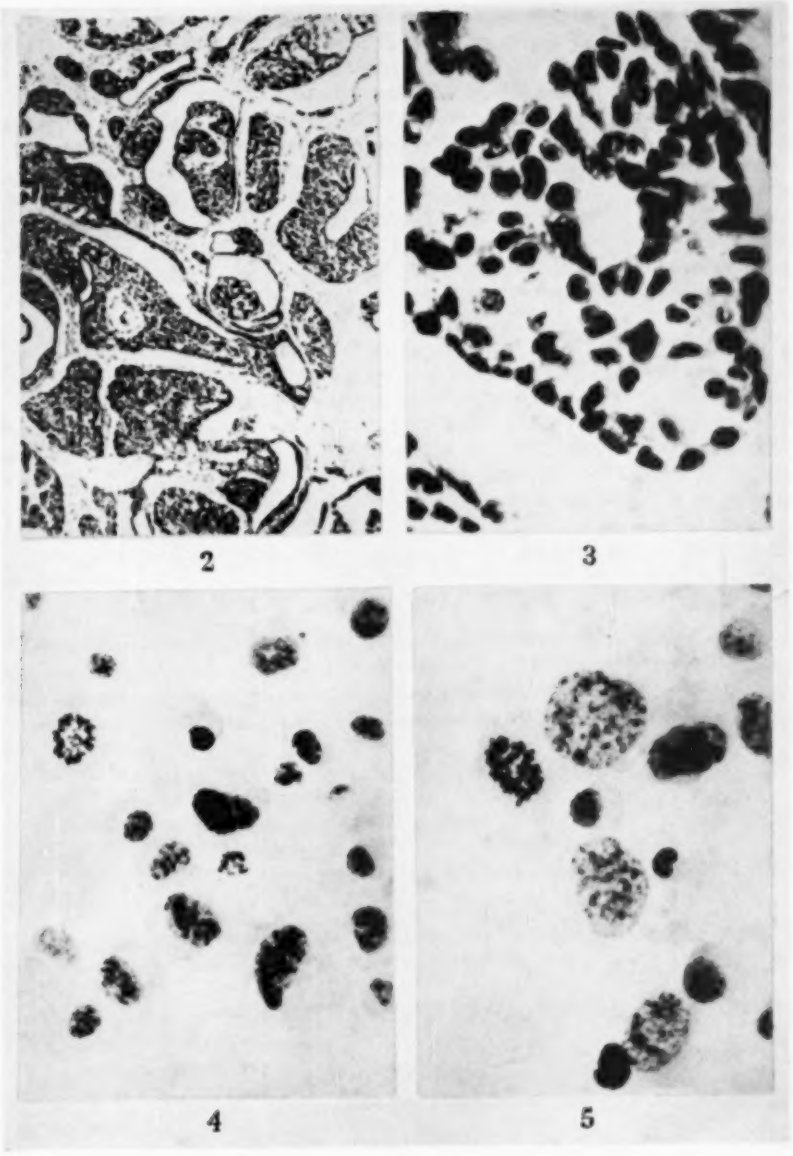
generation, there were great fluctuations of both factors in all the lines. However, once conversion had taken place there was a dramatic rise in tumour-cell content of the ascitic fluid and an associated decrease in host survival. This has been maintained during the subsequent period of transfer of the various lines.

EXPLANATION OF PLATE

FIG. 2.—Tumour No. 1. Solid polygonal cell mammary carcinoma (type M) from Balb/C+ mouse. H. and E. ×50.

FIG. 3.—High power view of tumour seen in Fig. 2. H. and E. ×460.

FIG. 4 and 5.—Neoplastic cells (with mitotic figures) of the ascites tumour derived from the above mammary carcinoma at the 30th transfer generation. Acetic-orcein. ×600.



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4 5

Biancifiori and Caschera.

DISCUSSION

Of the nine tumours tested, one spontaneous mucoid adenocarcinoma arising in a Balb/C+ mouse carrying the milk factor was successfully converted to an ascites tumour. It may or may not be significant that the only other tumour which seemed likely to convert (No. 3) arose also in this strain, though unlike the previous tumour it had been transplanted once prior to test and was of solid polygonal-cell type. The factors which determine the conversion are virtually unknown, but it may well be that tumours 6 and 7 would have converted at a later date had transfer been continued. The present tumour can rightly be called an ascites tumour as it conforms with Klein and Klein's (1955) definition of a tumour " in which active multiplication of free neoplastic cells and/or cell complexes can be shown to occur in the peritoneal fluid, leading to a high absolute and relative concentration of tumor cells, that is to say to a nearly pure culture ".

Klein and Klein (1955) tried, without any success, to convert 17 spontaneous mammary adenocarcinomas to the ascites type. They did, however, succeed in converting four to six *transplanted* mammary adenocarcinomas. These authors give a good summary of previous conversions of solid tumours to ascites tumours.

Klein and Klein (1951) found that there was an inverse relationship between the tumour cell content of the inoculum and the survival time of the hosts. They suggested that " large numbers of virulent tumor cells multiply freely in the peritoneal fluid, and their overwhelming effects kills the animals within a short time. This short survival time prevents the formation of more voluminous solid tumors ". In the present experiment this stage seemed to be reached at about the 30th transfer generation.

SUMMARY

An attempt was made to convert nine mammary carcinomas, five spontaneous and four transplanted, occurring in three strains of mice carrying the milk factor, to ascites tumours by repeated intraperitoneal transfer. The attempt was successful in regard to a mucoid adenocarcinoma occurring spontaneously in a female of the Balb/C+ strain.

The change from a combination of solid and liquid to completely liquid tumour took place in 5 different lines at the 33rd to the 37th generation. In the generations preceding the final stage there had been an increase in the tumour-cell content of the ascitic fluid and a tendency to decrease of the survival time of the host.

All the ascitic tumour lines continue to be maintained by intraperitoneal transfer.

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REFERENCES

DUNN, THELMA B.—(1953) In ' Physiopathology of Cancer '. Ed. F. Homburger and W. H. Fishman, London (Cassell & Co. Ltd.).

KLEIN, G. AND KLEIN, EVA.—(1951) *Cancer Res.*, **11**, 466.—(1955) *Ann. N.Y. Acad. Sci.*, **63**, 640.

OLIVI, MARIA, BIANCIFIORI, C. AND BARBIERI, G.—(1955) *Lav. Ist. Anat. Univ. Perugia*, **15**, 5.

VIRUS-CELL RELATIONSHIP IN KIDNEY TUMOURS INDUCED IN GOLDEN HAMSTERS BY THE MILL HILL POLYOMA VIRUS

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MILL HILL polyoma virus (M.H.P.) was isolated from the spleen of a leukaemic AK mouse in 1959 (Negroni, Dourmashkin and Chesterman, 1959). It has many properties in common with the Stewart and Eddy polyoma virus (Stewart, Eddy, Haas and Borgese, 1957; Stewart, Gochenour, Borgese and Grubbs, 1957; Eddy, Stewart, and Touchette, 1958; Stewart and Eddy, 1959) and, furthermore, rabbit antisera, prepared against each of these viruses, cross-react in the haemagglutination inhibition test (Negroni, unpublished).

High doses of M.H.P. injected into 1–5 day old hamsters produced kidney tumours in over 90 per cent of the animals together with vascular lesions and/or tumours of the liver, heart and lungs (Chesterman and Negroni, 1960, unpublished). From the kidney tumour cells M.H.P. virus could always be re-isolated in mouse embryo tissue cultures, but the serial transmission of the virus from hamster to hamster proved to be difficult. Only 5 out of 26 animals inoculated with cell-free extracts from kidney sarcoma showed tumours after an average incubation period of 219 days. This suggests that only a small amount of virus is present in the tumour. The purpose of this paper is to present data which confirm this and which clarify the cell-virus relationship in these kidney tumours. The kidney has been chosen in our studies because of the prevalence of tumours in this organ, and their early appearance after virus inoculation.

MATERIAL AND METHODS

Animals.—C3H mice are bred by brother-sister mating. Mice are killed on the 14th day of pregnancy and cultures prepared from the embryos. Golden hamsters are bred in a closed colony but not by brother-sister mating. They are inoculated with virus 1–5 days after birth, either by subcutaneous or intraperitoneal route.

Tissue culture.—Mono-layer tissue cultures from C3H mice or hamster kidney tumours are prepared in roller tubes by the method of Melnick (1955). The tubes are kept stationary for 1–3 days at 37° C., and then rolled at 37° C. The medium is 10 per cent calf serum, 0·5 per cent lactalbumin hydrolysate and 89·5 per cent Hanks solution plus antibiotics. The medium is changed twice a week.

Virus.—The virus is a tissue culture line passed serially with 0·1 ml. of medium containing 10^6–10^7 tissue culture infective doses (T.C.I.D.). The cultures are inoculated within one week of setting.

Haemagglutination.—Two-fold serial dilutions of virus in phosphate buffered saline are treated with 3–4 $\times$ 10^7 guinea-pig red blood cells, in the same medium, at 4° C. The end-point is taken as the last dilution showing complete haemagglutination.

Haemagglutination inhibition test.—Two-fold serial dilutions of inactivated sera are challenged with 8 haemagglutinating doses of virus at 30° C. for 1 hour and then left at 4° C. with 3–4 $\times$ 10^7 guinea-pig red blood cells. The end-point is taken to be the last dilution preceding complete haemagglutination.

Infectivity titration.—Ten-fold serial dilutions of virus are inoculated into groups of 7 day old tissue cultures of mouse embryo and incubated at 37° C. The medium is changed twice a week. The experiments are terminated 4 weeks after the inoculation. The 50 per cent end-point is calculated by the method of Reed and Muench (1938) on the basis of cytopathic changes and haemagglutination.

The haemagglutination test is carried out at each change of medium; at the end of the experiment homogenized tissue culture cells are added to the medium. The haemagglutination test was positive only when the tissue culture cells showed cytopathic changes.

EXPERIMENTS AND RESULTS

Relationship of dose to incubation period in mouse embryo fibroblasts (MEF)

The inoculation of MEF cultures with large amounts of MHP (10^6–10^7 T.C.I.D.) produces cytopathic changes in the cells which become apparent after 5 days. The period between infection and the detection of such changes increases as the amount of virus in the inoculum decreases.

Fig. 1 shows three experiments in which the time of appearance of the cytopathic changes is related to the number of infectious doses. The results with high doses are uniform but the appearance of cytopathic changes is scattered over a long period of time (2–3 weeks) with smaller doses.

Minces of kidney tumour cells, from hamsters inoculated with M.H.P. when 1–5 days old, were used to infect monolayer tissue cultures of mouse embryo cells. Cytopathic changes in these cultures were noticed in the 2nd or 3rd week after infection.

This is shown in Fig. 2 which refers to 7 isolations of virus from primary hamster tumours.

Attempts to reveal additional virus masked by antibody in the tumour

The long incubation period between infection and appearance of cytopathic changes, however, could not be taken as complete proof that only a small amount of virus is present in the tumours. Antibody in these tumours might be masking larger amounts of virus. This objection is sustained by the finding of high titres of antibody as measured by the haemagglutination inhibition test. Fig. 3 shows the results obtained in two experiments in which groups of 2–4 hamsters were killed at 3-day intervals after the inoculation. Sera from these animals were pooled and titrated for haemagglutination inhibition. Titres of 1 in 20,000 in the experiment in which 5-day old hamsters were inoculated and titres of 1 in 5000 in new-born hamsters, were obtained two weeks after inoculation.

Virus-induced hamster kidney tumours can be transplanted serially into adult hamsters—the sera from these animals contained no antibodies to the virus. Table I shows the results of the haemagglutination inhibition test with 12 sera from hamsters with transplanted tumours. The animals were chosen from various transplant generations between the first and the tenth. The sera

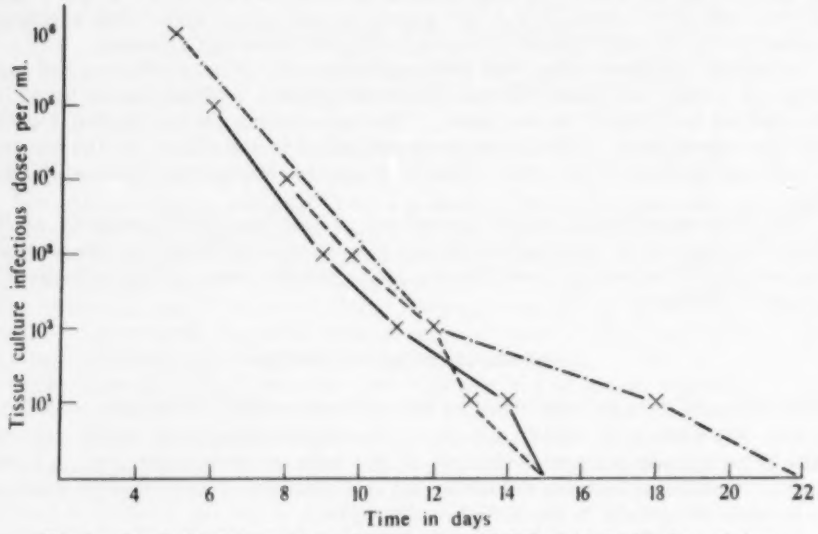

FIG. 1.—Correlation between time of appearance of C.P.E. and infecting dose of virus (three separate experiments).

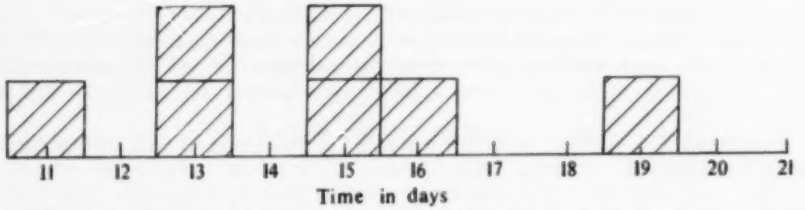

FIG. 2.—Time of appearance of cytopathic effect (C.P.E.) in mouse embryo tissue cultures infected with virus from hamster tumours.

Each square refers to one animal.

were only collected from animals showing tumours at the site of implantation. The interval between the inoculation and collection of sera varied therefore, according to the number of the transplant generation, 280 days in the first, and 14 days in the tenth. There was, however, uniformity in the result; the sera tested showed no antibody in the haemagglutination inhibition test. The absence of antibodies in the sera of adult hamsters with *transplanted* kidney tumours, compared with the high titres of antibodies in the sera of hamsters with *virus-*

induced tumours, further indicated that these tumours only contained a small amount of virus.

An experiment was devised, therefore, in which the effect of antibodies could be excluded. The results are summarized in Table II. 6 mg. of washed and

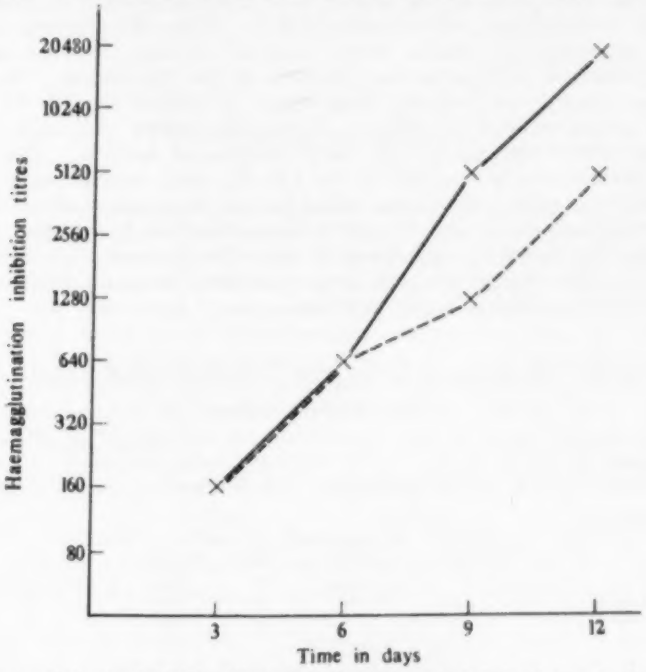

FIG. 3.—Haemagglutination inhibition titres of sera from hamsters killed at intervals after inoculation with virus.

——— 5-day-old hamsters.
– – – – 1-day-old hamsters.

TABLE I.—*Haemagglutination Inhibition Titres of Sera from Hamsters with Transplanted Tumours*

Transplant generation	Days after inoculation	Titre
Control	—	<100
1	280	<100
1	99	<100
1	70	<100
2	224	<100
2	273	<100
2	218	<100
2	62	<100
2	72	<100
3	43	<100
3	34	<100
3	49	<100
10	14	<100

centrifuged cells from a hamster kidney tumour was inoculated into mouse embryo fibroblasts. These showed cytopathic changes 16 days later. The supernatant from the above centrifugation, injected into mouse embryo fibroblasts, produced no cytopathic effects. From the same tumours mono-layer tissue cultures were prepared and grown in conditions identical to those used for mouse embryo fibroblasts infected with M.H.P. When the cultures were established the semi-confluent sheets were composed of large, fusiform cells which were well-preserved throughout the duration of the experiment. The medium from these cultures was serially diluted and inoculated on the 9th day into groups of mouse embryo fibroblasts. Cytopathic effect was only noticed in cultures inoculated with 0·1 ml. of 1 in 10 dilution of medium. The cells from the same cultures were trypsinised on the 15th day and counted under the microscope. Half was added, at various cell dilutions, to mouse embryo fibroblasts. The other half was frozen and thawed 5 times, and then inoculated into groups of mouse embryo fibroblasts separately at each dilution level. No difference was noted between the two groups and cytopathic effect was only found in cultures inoculated with 3×10^4 cells per inoculum.

TABLE II.—*Infectivity of Virus from Hamster Kidney Tumour Cells*

Hamster kidney tumours						
		Tissue culture				
Washed cells, 6 mg. ↓ M.E.F.*	Supernatant ↓ M.E.F.		Medium ↓ M.E.F.		Cells† ↓ M.E.F.	
	Dil.	C.P.E.	Dil.	C.P.E.	No.	C.P.E.
C.P.E. + ve 16 days	0	− ve	0	2/3	3×10^4	2/3
	10^{-1}	− ve	10^{-1}	1/3	3×10^3	0/3
			10^{-2}	0/3		

C.P.E. = cytopathic effect.
* Mouse embryo fibroblasts.
† Frozen and thawed, or intact cells.

Kidney virus titres before the development of macroscopic tumours

Two experiments were carried out to see whether multiplication of the virus occurred in the kidney before the tumours became established. Suckling hamsters inoculated when 5 days old in Experiment 1, and 1-day old in Experiment 2, were killed at three-day intervals, from the 3rd to the 12th day. Equivalent amounts of pooled kidney tissue, 8–32 mg. from 2–3 animals, were diluted serially, and 0·1 ml. was inoculated into groups of cultures of mouse fibroblasts at each dilution level. The results are shown in Table III. While a small amount of virus was present throughout the period of our experiment, there was, however, no significant difference in the amount of virus recovered from the kidney at any time after the inoculation.

DISCUSSION

Virus titrations carried out with tumours from hamster kidney show that only a small amount of virus is present in the tumour cells. This explains why cell-free preparations from tumours, when inoculated into hamsters, produced

TABLE III.—*Infectivity Titres of Virus from 8–32 mg. Kidney Tissue of Hamsters Killed at Intervals After Inoculation*

Days after inoculation	T.C.I.D. of M.H.P. in hamster kidneys	
	Experiment 1	Experiment 2
3	1	$10^{2.5}$
6	1	$10^{1.5}$
10	1	$10^{1.3}$
12	1	$10^{1.6}$

Experiment 1.—5-day-old hamsters.
Experiment 2.—1-day-old hamsters.

no tumours in one experiment or only a few in a second experiment after a long incubation period. Tissue cultures established from the tumours also contain a small amount of virus, and this shows clearly that antibodies are not masking larger amounts of virus. This is also indicated by the lack of antibody formation in hamsters with transplanted tumours. Multiplication of the virus in the kidney of the hamsters could not be demonstrated at any time after the inoculation. It may be concluded that these tumour cells are not virus producing; however, there can be no doubt that the virus is directly responsible for the primary changes in the cells which lead to the formation of the tumour. The very early appearance of this tumour in the kidney, where tumour cells in small groups are already present on the third day after the inoculation in some animals, precludes any sequence of initiation and promotion (Chesterman and Negroni, 1960, unpublished).

There are substantial differences between the results discussed in this paper and those found in cultures of mouse embryo tissue infected with polyoma. When tissue cultures of mouse embryo fibroblasts are infected with large amounts of polyoma virus, virus production starts on the second day, and reaches a peak on the fifth day after the inoculation. (Vogt and Dulbecco, 1960; Negroni, 1960). With smaller amounts of infectious virus the same peak of virus production is obtained but after a longer incubation period. The cells which seem more susceptible to the action of polyoma are the epithelial cells; the fibroblasts are apparently more resistant. If tissue cultures are infected with polyoma virus and then receive 24 hours later rabbit anti-polyoma antiserum the epithelial cells die and detach from the glass surface while the fibroblasts reconstitute the cell sheet by migration and multiplication (Negroni, 1960). Removal of antibodies, however, is promptly followed by cytopathic changes in these fibroblast cultures. In similar experiments carried out with kidney cells from suckling mice, the resistant fibroblasts did not show cytopathic changes after the removal of antibodies; moreover they did not show such changes after re-inoculation with a second dose of virus.

The destruction of mouse epithelial cells which probably occurs as a result of virus multiplication inside their nuclei (Banfield, Dawe and Brindley, 1959; Negroni, Dourmashkin and Chesterman, 1959; Dourmashkin and Negroni, 1959) does not occur in hamster kidney cells. In this lies the main difference between the hamster tissue and the mouse tissue culture mono-layer.

However, under either condition, there are virus-infected cells which do not produce virus. In the hamster, infection leads immediately to the formation of a

tumour ; in the mouse, the tumours arise after a longer latent period, and the factors which determine the time of appearance of these tumours remain to be discovered.

SUMMARY.

Mill-Hill polyoma (M.H.P.) virus produced kidney tumours in over 90 per cent of hamsters inoculated when newly born. Virus titrations with mixed hamster kidney tumours showed that only a small amount of virus was present in the tumour cells. Tissue cultures established from the tumours also contained a small amount of virus showing that antibodies were not masking larger amounts of virus. No rise of antiviral antibodies was detected in the serum from hamsters with transplanted kidney tumours. The kidney tumours may occur as early as the 3rd day after inoculation of M.H.P. virus. This indicates that virus is directly responsible for the primary change in the cells leading to the formation of a tumour.

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REFERENCES

Banfield, W. G., Dawe, C. and Brindley, D. C.—(1959) *J. nat. Cancer Inst.*, **23**, 1123.
Dourmashkin, R. R. and Negroni, G.—(1959) *Exp. Cell Res.*, **18**, 573.
Eddy, B. E., Stewart, S. E. and Touchette, R.—(1958) *Proc. Amer. Ass. Cancer Res.*, **2**, 294.
Melnick, J. L.—(1955) *Ann. N.Y. Acad. Sci.*, **61**, 754.
Negroni, G.—(1960) *Exp. Cell Res.*, in press.
Idem, Dourmashkin, R. R. and Chesterman, F. C.—(1959) *Brit. med. J.*, ii, 1359.
Reed, L. J. and Muench, H.—(1938) *Amer. J. Hyg.*, **27**, 493.
Stewart, S. E. and Eddy, B. E.—(1959) ' Perspectives in Virology '. Ed. M. Pollard. New York (Wiley), p. 242.
Idem, Eddy, B. E., Haas, V. H. and Borgese, N. G.—(1957) *Ann. N.Y. Acad. Sci.*, **68**, 419.
Idem, Gochenour, A. M., Borgese, N. G. and Grubbs, G. E.—(1957) *Virology*, **3**, 380.
Vogt, M. and Dulbecco, R.—(1960) *Proc. nat. Acad. Sci., Wash.*, **46**, 365.

STUDIES ON THE ONCOGENIC ACTIVITY OF THE TORONTO STRAIN OF POLYOMA VIRUS

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POLYOMA virus was first isolated by Stewart and Eddy from leukaemic mice (Stewart *et al.*, 1957) and was subsequently shown to induce multiple tumours after inoculation into newborn mice, hamsters, rats and rabbits. This virus has been designated SE polyoma virus. A virus with similar properties was subsequently obtained from mouse mammary carcinoma tissues by McCulloch *et al.* (1959). This virus is antigenically related to SE polyoma, but there is a lower pathogenicity for mice (Howatson *et al.*, 1960). For convenience we refer to it as T (Toronto) polyoma. Another polyoma-like virus (MH polyoma) has also been isolated by Negroni, Dourmashkin and Chesterman (1959), which is related antigenically to T polyoma (Stoker and Macpherson, unpublished). It appears from the results of Rowe *et al.* (1959*b*) that strains of polyoma virus may be common commensals in laboratory mice, even in stocks with a low tumour incidence.

Polyoma virus, like Rous sarcoma virus, has several advantages for investigating the nature of viral oncogenesis. It has a short incubation period and high efficiency of tumour induction; it grows in mouse embryo and other cells in culture giving high titre virus stocks; the cytopathic effect induced in mouse cells permits assay by the plaque technique (Dulbecco and Freeman, 1959; Winocour and Sachs, 1959). The virus agglutinates red blood cells, so a simple method is also available for enumerating total virus particles (Eddy *et al.*, 1958; Kahler *et al.*, 1959). Finally, the virus is stable under ordinary physiological conditions.

This paper describes studies in dosage requirements for induction of tumours by polyoma virus in hamsters. T polyoma virus has been used because of its high pathogenicity for these animals.

METHODS

Tissue cultures

Mouse embryo cells were obtained by trypsinization of whole embryos from Swiss mice. Four million cells were added to each 60 mm. Petri dish, and incubated in modified Eagle's medium with twice the normal concentration of amino acids and vitamins, and 10 per cent calf serum. This serum was replaced by 5 per cent horse serum after inoculation of virus for preparation of stocks. Either primary or secondary cultures (after re-dispersal with trypsin) were used, and all cultures were incubated in a mixture of air and added CO_2 sufficient to maintain the pH at 7·2–7·4.

Virus stock

T polyoma virus, derived from the strain isolated by McCulloch *et al.* (1959) was kindly sent from Toronto by Dr. L. Siminovitch as infected tissue culture fluid. Virus stock (Stock 1) was prepared from monolayer cultures of mouse embryo cells inoculated with either 0·01 or 0·1 plaque forming units (PFU) per cell. Medium was collected from cells during cytopathic changes on subsequent days, pooled after a preliminary titration of haemagglutinin centrifuged at 250 g for 5 minutes and stored at −70°. Another stock (Stock 7) was obtained from virus after cloning by three cycles of plaque purification. This was concentrated by homogenization of infected cells in a small volume of distilled water, and the virus was partially purified by exposing the crude homogenate to 3 cycles of extraction with fluorocarbon.

Plaque assay

The technique used was a modification of that described by Dulbecco and Freeman (1959) and Winocour and Sachs (1959). Confluent primary or secondary cultures of mouse embryo cells in 60 mm. Petri dishes were used. The best secondary mono-layers were obtained when the cells from confluent primary cultures were put back into the same dishes.

After removal of the medium, the appropriate dilution of virus was added in 0·2 ml. volumes to each plate. After adsorption for 2 hours at 37° C., an overlay was added to each, comprising 6 ml. of medium with 2·5 per cent horse serum and 0·9 per cent agar (Difco Bacto). The cultures were fed with 3 ml. of the same overlay mixture on the 4th and 8th days. Neutral red 0·01 per cent was incorporated in the final overlay.

Plaques appeared as pale areas with diffuse borders which contained stained and healthy-looking cells as well as degenerate cells (Dulbecco and Freeman, 1959). Fresh plaques appeared on successive days, and final counts were not made until the number remained more or less stationary, usually by the fourth day after staining for secondary cultures, but not until the eighth day for primary cultures.

The number of plaque forming units was determined by counts on at least 3 plates with 10 or more plaques per plate and a standard suspension of virus which had been titrated many times and under varying conditions, was included in each assay to check the sensitivity of the batch of cells.

Some secondary monolayer cultures underwent a spreading degeneration after ten days or more under the overlay mixture. On affected plates this appeared as a gradually increasing and sharply demarcated area of complete cell degeneration, which on successive days sometimes involved a large proportion of the culture and thus obscured the plaques. The cause of this degeneration has not been determined. Though increased by additional neutral red, it is apparently not due to photosensitization (Green and Opton, 1959).

Haemagglutination titrations

After heating at 37° for 15 minutes to reduce inhibitor activity, serial two-fold dilutions of virus suspensions were made in cold phosphate buffered saline (PBS of Dulbecco and Vogt, 1954) in 0·2 ml. volumes in perspex haemagglutinating trays. To each dilution was added 0·2 ml. of cold 0·5 per cent guinea-pig erythro-

cytes diluted in buffer. The mixtures were left at 4° for 2–30 hours before reading the pattern of deposited cells. The smallest quantity of virus causing partial agglutination was taken as one haemagglutinating unit (HAU) and was expressed as the reciprocal of the dilution.

Antibody was titrated by inhibition of haemagglutination. Eight HAU of virus in 0·2 ml. volumes were added to serial twofold dilutions of antiserum in 0·2 ml., followed by 0·2 ml. of 0·5 per cent guinea-pig erythrocytes. The antibody titre was taken as the highest dilution of antiserum showing partial inhibition of haemagglutination, but titres of below 1/160 were taken to be due to inhibitor rather than antibody.

Animal inoculations

Pregnant Syrian hamsters were delivered to the laboratory from a dealer 3–7 days before parturition. The young from these mothers were inoculated 24–48 hours after birth by injection of 0·1 ml. of virus suspension, either intra-abdominally or subcutaneously into the back. The animals in each litter received the same inoculum. There was no obvious variation in results between different litters from the same breeder receiving the same inoculum. Animals which disappeared, mostly in the first week, and a few which died later without detectable tumours were not counted in the results.

Tests for polyoma antibody in the breeding hamsters from the dealer concerned have not shown evidence of natural infection in the stock. The hamsters were observed daily or twice daily, and when sick or dead were removed for post mortem examination. Specimens were fixed in 10 per cent formal saline and after sectioning were stained with haematoxylin and eosin. The skull contents, spinal cord and skeletal systems were not examined.

Pregnant rats were also obtained from a dealer and the litters inoculated and observed in the same way. Weanling Swiss mice from a dealer were inoculated with 0·1 ml. quantities of virus suspension for antibody production tests (Rowe *et al.*, 1959*a*). The mice were killed 21 days later and blood was collected separately from each animal for haemagglutination inhibition tests.

RESULTS

Tumour production in hamsters

Stock 1, which was used for most of the animal inoculations, contained $10^{6.88}$ PFU and $10^{3.50}$ HAU per ml. When 0·1 ml. of undiluted stock containing $10^{5.88}$ PFU was inoculated intra-abdominally into newborn hamsters, they died between ten and fifteen days later (Fig. 1). All showed diffusely enlarged pale kidneys and cysts containing blood in the liver. Microscopic examination showed the renal medulla to be almost completely replaced by spindle cells, resembling a spindle cell sarcoma. A thin, compressed, cortex remained relatively unaffected. The livers showed a patchy centrilobular haemorrhagic necrosis and large spaces filled with blood. Some of the animals apparently died as a result of intra-abdominal haemorrhage from the liver ; others showed no haemorrhage and perhaps died from renal or hepatic failure.

When 10^{7} PFU were inoculated from the higher titre Stock 7 the hamsters died even earlier, between seven and twelve days after inoculation, showing renal tumours, hepatic necrosis and haemorrhage.

With lower doses of virus from Stock 1, there was an increasing delay before death. Fig. 1 shows the relationship between virus dose and the interval before death from tumour. When $10^{2.88}$ or $10^{1.88}$ PFU were inoculated, some animals revealed no macroscopic evidence of tumours when killed 23 weeks after inoculation.

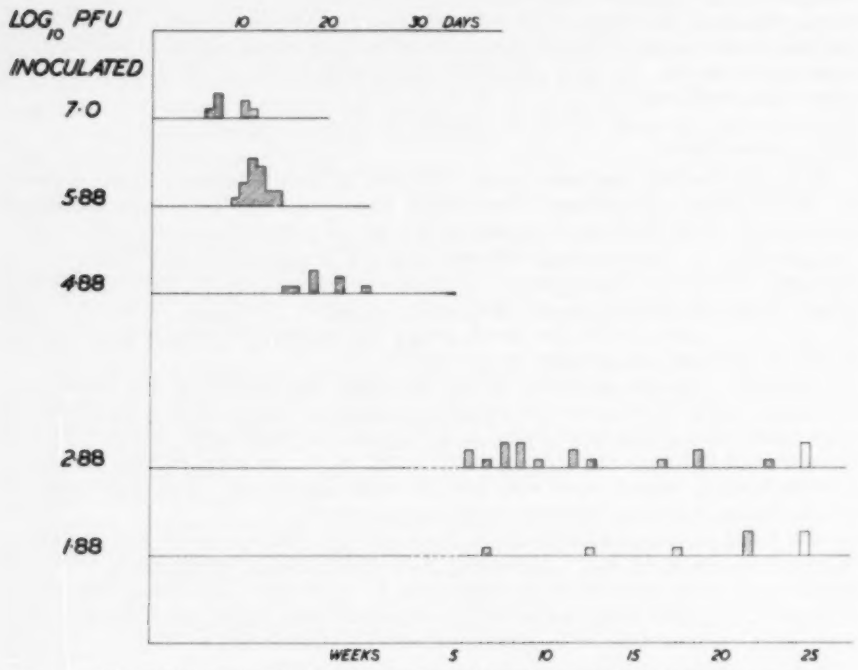


FIG. 1.—Interval before death after intraperitoneal inoculation of 1 to 2-day-old hamsters with varying doses of T polyoma virus. Shaded columns indicate hamsters dying with tumours. Open columns indicate survivors without tumours.

Types of tumour

The only lesions observed macroscopically in animals dying in the first two weeks after intraperitoneal inoculations were those of the kidney and liver. Detailed histological examination of other organs was not carried out however.

Hamsters inoculated with $10^{4.88}$ PFU or less, dying in the third week or later, developed tumours in certain other sites as well, particularly in the myocardium, and in the peritoneal cavity where they appeared as multiple polyps on the surface of the gut. More rarely, tumours were found in the anterior mediastinum, suprarenal, testis, lung, and subcutaneous tissue. All these tumours histologically resembled spindle cell sarcomata.

Subcutaneous instead of intraperitoneal inoculation of $10^{5.88}$ PFU resulted in tumours of the kidney, and myocardium, and hepatic necrosis and haemorrhage. Even though this route of inoculation was followed by deaths in the third or

subsequent weeks, no tumours of gut wall were seen, so it is concluded that these are the results of direct intra-abdominal inoculation rather than spread via the blood stream.

The blood filled cysts on the liver macroscopically resembled cavernous haemangio-endotheliomata, but microscopic examination showed no endothelial proliferation. Indeed, the cysts sometimes appeared to be lined with parenchymal cells rather than vascular endothelium.

Tumours and necrosis produced by clonal stocks of virus

It was possible that the different tumours and more particularly the necrotic lesions of the liver, might be produced by a mixture of different types of virus particle in the inoculum. Accordingly clones of virus derived from single particles were obtained by three consecutive plaque isolations. Two such clones were respectively inoculated into hamster litters, and each produced typical renal, myocardial and gut wall tumours as well as hepatic necrosis and haemorrhage. It therefore appears that a single virus particle carries the genetic potentiality to initiate several of the different lesions.

Time of appearance of tumour cells

Since hamsters inoculated with large doses of virus died in less than two weeks with massive renal tumours, an attempt was made to detect the first appearance of tumour cells.

Eighteen hamsters in 3 litters were inoculated intraperitoneally with $10^{5.88}$ PFU of virus Stock 1, and pairs of animals were killed subsequently, 2, 4, 7, 9 and 11 days after inoculation. Both kidneys from each animal were taken for macroscopic examination. As controls, hamsters were inoculated with tissue culture medium from uninfected mouse cells, and single animals were killed for removal of kidneys at the same successive time intervals.

Two days after inoculation of the virus no difference could be seen between control and infected kidneys. Four days after inoculation however, clusters of abnormal cells were clearly seen in the kidneys from the infected hamsters (Fig. 2 and 3). These cell clusters were in the region of the collecting tubules near the cortico medullary junction. They lay in the interstitial region between the tubules and each contained 20 to 30 large polygonal cells with pale vesicular nuclei. Mitotic figures were present in 3 per cent of these cells, compared to 0·5 per cent of the cells in the intervening normal regions. No such clusters of cells were seen in the kidneys from the control animals killed at the same or any age, nor in serial sections of kidneys from four additional normal hamsters of the same age.

Seven days after inoculation, larger foci of the same type were present. After nine days, the regions with abnormal cells were almost confluent and the cells themselves were more elongated or spindle-like. Eleven days after inoculation, the kidneys were much enlarged and except for a thin strip of cortex, the renal tissue was replaced by spindle cells (Fig. 4).

Extensive degeneration of the interstitial cells with intranuclear inclusions and accumulated cell debris, as described by Ham *et al.* (1960) was not observed to be a feature of the early stages in this experiment.

Quantitative aspects

It is clear from Fig. 1 that the interval until death, or other obvious manifestation of tumours in the hamsters, is increased as the dose of virus is reduced. Thus a determination of the true minimum tumour producing dose should entail observation for the whole life span of the hamster to detect any late developing tumours following inoculation of limit doses.

This ideal was not realized, but Table I shows the proportion of hamsters developing macroscopic tumours during two periods of observation after inoculation with varying doses of virus.

TABLE I.—*Proportion of Newborn Hamsters and Rats Subsequently Developing Tumours, or Weanling Mice Subsequently Developing Antibody, After Inoculation with Varying Doses of T Polyoma Virus*

Inoculum		Tumours induced in :			Antibody production in mice (21 days)
		Hamsters		Rats	
Dilution factor	(PFU) Log 10	(63 days)	(165 days)	(175 days)	
0	5·88	19/19*	..	2/3	..
10^1	4·88	8/8	..	0/8	..
10^2	3·88	3/3	..	4/10	..
10^3	2·88	3/4	18/21	0/5	8/8
10^4	1·88	1/4	6/9	0/4	8/8
10^5	0·88	0/5	..	0/6	8/8
10^6	−0·88	..	..	..	7/7
10^7	−1·88	..	..	..	3/8
Controls†		0/7	0/8	0/9	0/8

* Number of animals with tumours/number observed for time given. Last column gives number of weanling mice developing antibody after 21 days/number tested.

† Controls were inoculated with medium from, or with extracts of, virus free cells.

When the experiment was terminated at 63 days after inoculation, the apparent ID50 was $10^{2.38}$ or 240 PFU. This corresponds to 340 PFU per tumour producing dose if the Poisson equation applies. When the hamsters were left for 165 days, however, a higher proportion developed tumours. The ID50 could not be measured but the figures suggest a tumour producing dose of 75 PFU or less.

The focal distribution of abnormal cells seen in the kidneys 4 days after inoculation of hamsters with large doses of virus raised the possibility that each focus was a clone of cells originating from a single initial virus cell interaction. If so, the number of foci should be proportional to virus dose.

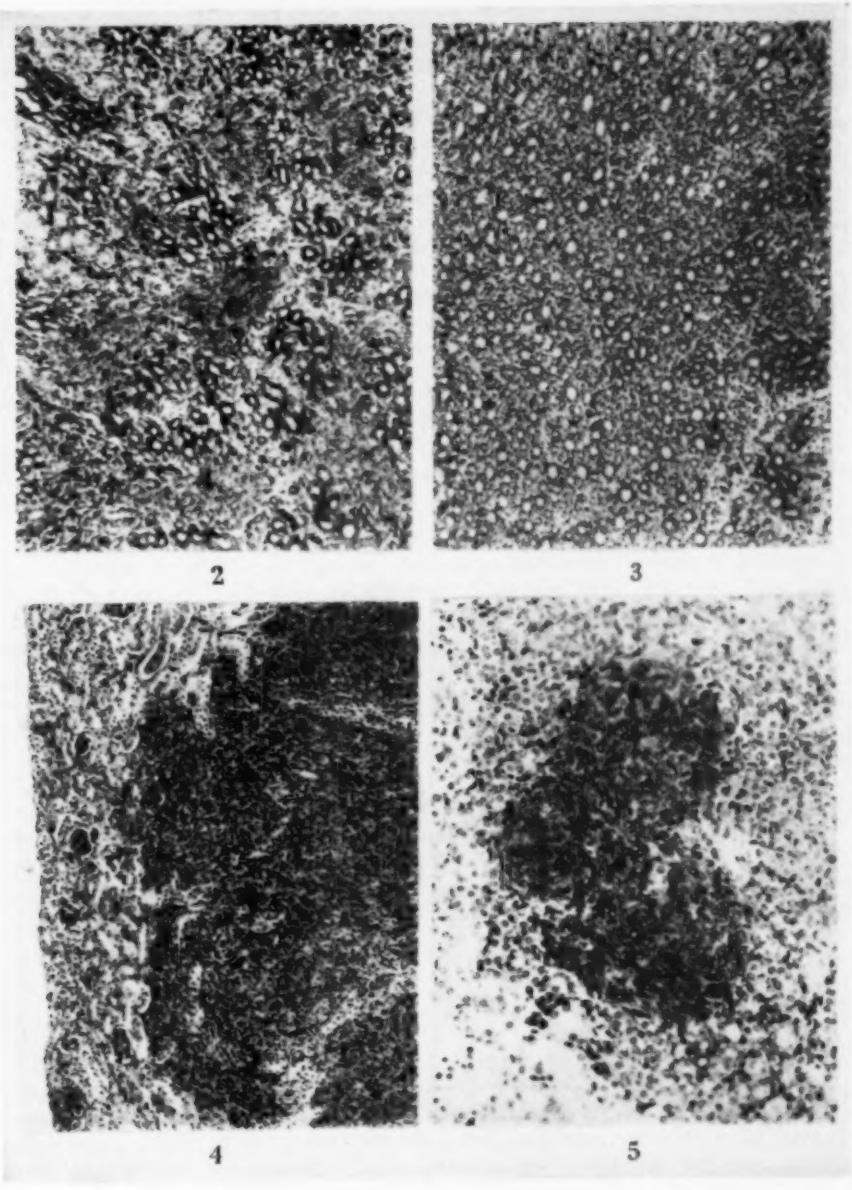
EXPLANATION OF PLATE

FIG. 2.—Tubule region of kidney from baby hamster 4 days after inoculation with $10^{5.88}$ PFU of T polyoma virus, showing foci of abnormal cells. Haematoxylin and eosin ×75.

FIG. 3.—Tubule region of kidney from normal hamster of same age as that shown in Fig. 2, for comparison. Haematoxylin and eosin × 75.

FIG. 4.—Part of medulla and cortex of kidney from baby hamster 11 days after inoculation with $10^{5.88}$ PFU of T polyoma virus, showing replacement of medulla by spindle cells. Haematoxylin and eosin ×75.

FIG. 5.—Single focus of tumour cells in kidney from baby hamster 9 days after inoculation with $10^{2.88}$ PFU of T polyoma virus. Haematoxylin and eosin ×150.



2 3

4 5

By assuming that the foci were distributed evenly throughout the medulla, it was estimated from counts of foci in a single section of each kidney, that approximately 10^3 foci per kidney resulted from inoculation of $10^{5.88}$ PFU. For more accurate counts, 12 hamsters were each inoculated with $10^{2.88}$ PFU and 10 hamsters were inoculated with $10^{1.88}$ PFU. They were all killed either 9 or 10 days later, and both kidneys from each animal were fixed and serially sectioned. (Only one kidney was obtained from each of two hamsters inoculated with $10^{2.88}$ PFU. These two single kidneys have been taken as coming from one animal, making a total of 11 kidney pairs in the batch.)

The serial sections were examined with a dissecting microscope and foci of tumour cells easily recognized and counted (Fig. 5). The diameter of the foci were 100–200 μ and were such that examination of a total of 30 or more regularly spaced sections through each kidney was sufficient.

The results shown in Table II show a correlation between numbers of foci and dose of virus at the two dilutions tested and agree well with the rough estimate of 10^3 foci per kidney after inoculation with $10^{5.88}$ PFU (Ratio PFU/focus = 375). The observed frequency distribution of foci in the group inoculated with $10^{2.88}$ PFU (Table III) agrees best with the theoretical distribution for an average of 1·7 focus forming particles per dose, calculated according to the Poisson formula, or 440 PFU per renal focus.

TABLE II.—*Number of Foci of Tumour Cells Seen in Serial Sections of Kidneys 9–10 Days After Inoculation of Newborn Hamsters with Indicated Dose of Virus*

Inoculum: Dilution factor	Inoculum: PFU Log 10	Number of foci in pairs of kidneys	Total foci	Mean per animal	Ratio PFU/focus
10^2	2·88	00 00 01 01 01 02 02 12 22 22 24	24	2·2	340
10^4	1·88	00 00 00 00 00 00 00 00 00 02 —	2	0·2	375

TABLE III.—*Frequency Distribution of Renal Foci per Animal Compared with Theoretical Distribution from Poisson Equation*

	Number of renal foci per animal: 0	1	2	3	4	5	6	7
Hamsters with number of renal foci shown	2	3	2	1	2	0	1	0
Observed distribution	0·18	0·27	0·18	0·09	0·18	0	0·09	0
Theoretical distribution for 1·7 focus forming units per inoculum	0·18	0·31	0·26	0·15	0·06	0·01	<0·01	<0·01

Litter mates of the hamsters which were killed 9 days after inoculation were observed for up to 165 days to see if the proportion dying with obvious renal tumours corresponded to the proportion with foci of tumour cells at 9–10 days. Table IV shows that the number finally showing massive renal tumours is as expected from the number with early foci. This means that the foci do not retrogress, but continue to develop into typical tumours. Since the proportion with involvement of one kidney only was the same at 9 days, and at death after

several months, it also suggests that tumour cells do not metastasize in the unaffected kidney.

TABLE IV.—*Comparison of Number of Kidneys Involved in Each Inoculated Hamster at 9–10 Days and in Subsequent Observation Period*

Inoculum		Renal foci at 9–10 days in :			Renal tumours present at 165 days or earlier death		
Dilution factor	PFU log 10	None	One kidney	Both kidneys	None	One kidney	Both kidneys
10^3	2·88	2	5	4	6	7	4
10^4	1·88	9	1	0	7	2	0

Further evidence against metastases comes from the small total number of tumours found in the various organs of each animal after small doses of virus. Table V gives the frequency distribution of macroscopic tumours in all organs examined. It shows that the number is roughly related to dose of virus and that most animals dying after inoculation of a small quantity of virus only developed a single tumour. It should be made clear, however, that a detailed histological search was not made, nor were the skull contents, spinal cord, or bones examined.

TABLE V.—*Frequency Distribution Showing Number of Tumours Observed Macroscopically at All Sites Examined in Hamsters Inoculated with T Polyoma Virus*

Inoculum		Number of tumours per animal					
Dilution factor	PFU log 10	0	1	2	3	4	5
10^3	2·88	3	4	7	3	2	0
10^4	1·88	3	5	1	0	0	0

These results suggest that a single particle of virus can initiate a focus of tumour cells in the kidney, and that this will probably occur if some 300–500 PFU are inoculated intra-abdominally. It should be noted that the dose required to produce a tumour in any site is not much less, and this suggests a relatively high susceptibility of the renal tissue.

Tumour production in rats

When 24–48 hour old rats were inoculated with T polyoma virus, they developed renal tumours resembling those seen in hamsters. More rarely tumours were present in lungs, heart and abdominal wall. From the proportion of rats with tumours shown in Table I it would appear that these animals are less susceptible and unsatisfactory for titration of oncogenic activity.

Mouse antibody production test

Rowe *et al.* (1959*a*) have shown that weanling mice develop haemagglutinin inhibiting antibodies if inoculated with small amounts of virus and thus may be

used for virus titration by determination of the minimum quantity of virus suspension needed to produce antibody in individual mice.

The results of a titration of Stock 1 by this method are included in Table I and confirm the high sensitivity. A satisfactory end point was not obtained through omission of a sufficiently low dose of virus. From the proportion positive in the group, given a mean of 0·075 PFU per animal, the minimum amount required to produce antibodies is apparently about 0·15 PFU.

DISCUSSION

The observation of very early tumour development after newborn hamsters are inoculated with undiluted suspensions of T polyoma virus agrees with the reports of McCulloch *et al.* (1959), Axelrad *et al.* (1960) and Ham *et al.* (1960). Not only do the animals die with massive renal tumours 1–2 weeks after inoculation, but foci of large abnormal cells with a high mitotic index can be seen between the developing tubules of the kidney as early as 4 days after inoculation.

In the series reported in this paper, the appearance of the tumour cells was not preceded by obvious degenerative changes in the interstitial cells between the tubules as described by Ham *et al.* (1960) but such changes may have been missed by infrequent sampling. Marked centrilobular haemorrhagic necrosis of the liver was indeed present however, in all animals exposed to high doses of virus, and death was often due to haemorhage from the characteristic blood filled cysts in the liver.

As might be expected reduction in virus dosage greatly delays the death or other obvious manifestation of tumour formation and this interferes with titrations for oncogenic activity. The focal distribution of the early renal lesions suggested that each group of tumour cells might be a clone initiated by a single virus particle. Though numbers are small, the counts of foci showed a fair agreement with virus dose, and, at high dilution, an approximation to Poissonian distribution. This relationship would not apply if some of the foci were initiated by virus which multiplied in the animal after inoculation, nor if tumour cells had formed secondary foci. The relatively constant size of the foci at 9 days also implies a nearly simultaneous origin.

If, as suggested by these results, the foci are clones of tumour cells, each initiated by infection with a single virus particle, one must ask if it is possible for the foci to reach the size observed 4 days after infection. Counts on 12 foci showed an average of 26 cells visible in the sections. Assuming that foci are spheres and that the sections are approximately equatorial, the number of cells per focus can be calculated from the section thickness and size of the cells, resulting in a mean of 65 cells per focus. This would result from 6 divisions from a single cell in 4 days, or a division time of 16 hours. The minimum division time of cells *in vivo* is not known, but 16 hours would correspond to the division time for many cell types growing in optimum conditions *in vitro*, including hamster kidney cells transformed by polyoma virus (Macpherson, unpublished). The mitotic index of the cells in the foci was 3 per cent (prophase not counted) giving 29 minutes for metaphase to telophase, which is also an acceptable figure. It is therefore concluded that the foci could have developed from single initially infected cells.

From the results, it appears that the foci of tumour cells do not retrogress. Despite the extremely active sarcoma-like growth of the early tumour cells however, the frequency of single tumours in animals dying many months after inoculation with minimum doses of virus suggests that the tumours do not metastasize. If so, the multiple tumours developing after large doses of virus are due to primary infection of the different sites, rather than secondary migration of tumour cells.

Though one virus particle can probably initiate a tumour, it is clear that many particles must be inoculated before this is likely to occur. Whether or not all particles are competent to induce tumours is not known, but there would obviously be a large wastage, quite apart from back leakage of the inoculum, since susceptible cells apparently occur in only a limited number of tissues. In terms of plaque-forming particles, several hundred must be inoculated to initiate a tumour in the kidney, but less than a hundred may be sufficient to start a tumour at any site (though appearance of such a tumour may take 5 months). Less than one plaque-forming particle is sufficient to infect a mouse so as to produce antibodies, and though more laborious and less accurate than plaque assay, this is still the most sensitive form of infectivity titration. None of these methods of titration measures total physical particles, however. From the haemagglutinin titres and the large numbers of particles seen by electron microscopy of viral suspensions (Wildy *et al.*, 1960) it is obvious that the number of physical particles which constitute the minimum oncogenic dose must be several orders of magnitude higher than the number suggested by infectivity titrations.

Rowe *et al.* (1959*a*) have already drawn attention to the difficulties involved in accurate measurement of the oncogenic properties of polyoma virus. Renal tumour focus counts as described here may provide an improved, though laborious method of titration, but are still subject to the many variables inherent in *in vivo* systems.

It is to be hoped that the virus induced transformation of cells *in vitro* described by Vogt and Dulbecco (1960) and Sachs and Medina (1960), will now provide the controlled conditions necessary for quantitative studies at the cellular level.

SUMMARY

Studies were carried out on the oncogenic activity of the Toronto strain of polyoma virus in hamsters. Foci of tumour cells appeared in the kidneys of newborn hamsters 4 days after inoculation with $10^{5.88}$ PFU. The animals died with massive renal spindle cell sarcomata and hepatic necrosis in 1–2 weeks. Reducing the dose of virus delayed the deaths and permitted development of tumours in heart and peritoneum and more rarely in suprarenals, lungs and testes and subcutaneous tissue. Inoculation of cloned virus showed that potentiality to form tumours in many sites and to cause hepatic necrosis is inherent in a single virus particle.

Counts of foci of tumour cells in the kidneys were roughly proportional to the virus dose and suggested that each focus constituted a clone of tumour cells initiated by a single virus cell interaction. Inoculation of about 400 plaque-forming units of virus was necessary to initiate one tumour focus in the kidney. Despite the early rapid invasion of the kidney there was no evidence that the tumours formed metastases.

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REFERENCES

Axelrad, A. A., McCulloch, E. A., Howatson, A. F., Ham, A. W. and Siminovitch, L.—(1960) *J. nat. Cancer Inst.*, **24**, 1095.

Dulbecco, R. and Freeman, G.—(1959) *Virology*, **8**, 396.

Idem and Vogt, M.—(1954) *J. exp. Med.*, **99**, 167.

Eddy, B. E., Rowe, W. P., Hartley, J. W., Stewart, S. E. and Huebner, R. J.—(1958) *Virology*, **6**, 290.

Green, R. H. and Opton, E. M.—(1959) *Proc. Soc. exp. biol. N.Y.*, **102**, 579.

Ham, A. W., McCulloch, E. A., Axelrad, A. A., Siminovitch, L. and Howatson, A. F.—(1960) *J. nat. Cancer Inst.*, **24**, 1113.

Howatson, A. F., McCulloch, E. A., Almeida, J. D., Siminovitch, L., Axelrad, A. A., and Ham, A. W.—(1960) *Ibid.*, **24**, 1131.

Kahler, H., Roew, W. P., Lloyd, B. J., and Hartley, J. W.—(1959) *Ibid.*, **22**, 647.

McCulloch, E. A., Howatson, A. F., Siminovitch, L., Axelrad, A. A. and Ham, A. W.—(1959) *Nature*, **183**, 1535.

Negroni, G., Dourmashkin, R., and Chesterman, F. C.—(1959) *Brit. med. J.*, ii, 1359.

Rowe, W. P., Hartley, J. W., Estes, J. D. and Huebner, R. J.—(1959*a*), *J. exp. Med.*, **109**, 379.

Idem, Hartley, J. W., Law, L. W. and Huebner, R. J.—(1959*b*) *Ibid.*, **109**, 449.

Sachs, L. and Medina, D.—(1960) *Nature* (in press).

Stewart, S. E., Eddy, B. E., Gochenour, A. M., Borghese, N. G. and Grubbs, G. E.—(1957) *Virology*, **3**, 380.

Wildy, P., Stoker, M. G. P., Macpherson, I. A. and Horne, R. W.—(1960) *Ibid.* **11**, 444.

Winocour, E. and Sachs, L.—(1959) *Ibid.*, **8**, 397.

Vogt, M. and Dulbecco, R.—(1960) *Proc. nat. Acad. Sci., Wash.*, **46**, 365.

A HISTOCHEMICAL STUDY OF THE EARLY STAGES OF CARCINOGENESIS IN RAT LIVER: LOCALIZATION OF FLUORESCENT CARCINOGEN AND CHANGES IN SUCCINIC DEHYDROGENASE ACTIVITY

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THERE are two major theories concerning liver carcinogenesis induced by azo-dyes. According to the first, the protein-deletion hypothesis (Miller and Miller, 1955) the dye becomes bound to a soluble cytoplasmic protein which is then "lost", in that it is not found in the tumour cells. The second (Elson and Hoch-Ligetti, 1945; Elson, 1958) holds that the more important phenomenon is the damage to the Krebs cycle produced by metabolites that inhibit succinic dehydrogenase.

It seemed of interest to test the localization and effect on succinic dehydrogenase of a structurally similar compound, 4-dimethylaminostilbene, which also produces cholangiomata when injected into rats (Elson, 1952). The advantage of this carcinogen is that it is naturally intensely fluorescent, so that if the whole molecule became bound to a cytoplasmic protein, as in the first theory, it might be seen even in very low concentration by direct fluorescence microscopy. Moreover, by the use of the cryostat microtome, the determination of succinic dehydrogenase activity has been made semi-quantitative, as checked by corresponding estimation on larger pieces of tissue by means of the Thurnberg tube method. Thus both hypotheses are open to direct histochemical investigation.

MATERIALS AND METHODS

1. *Animals and diet*

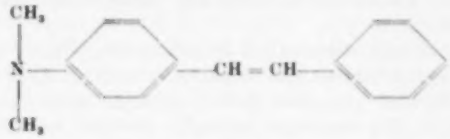
Albino Wistar male rats about 150 g. in weight were injected with 4-dimethylaminostilbene. They, and control animals, were fed on a specially prepared diet containing 5 per cent protein and an additional 0·4 per cent methionine (Diet C; Elson, 1952). Some rats, designated "normal animals" were fed on a well-balanced diet (M.R.C. rat diet B.41).

They were killed by placing them under a funnel through which nitrogen was passed from a cylinder at a rate of over 1 litre per minute.

2. *The carcinogen*

The 4-dimethylaminostilbene was synthesized by R. W. Baldwin; it was a dull yellow flaky crystalline substance, with a melting point between 148·5–

149° C. It was used as a 1 per cent solution in arachis oil. The structural formula of 4-dimethylaminostilbene is as follows :



3. *Injection and dose*

(a) *Intraperitoneal.*—The normal and the control animals (designated animals A_N and A_C) received no injections. One of the other rats was injected intraperitoneally with 0·5 ml. of arachis oil alone and was killed half an hour later. The remaining animals were given 0·5 ml. of the 1 per cent solution of 4-dimethylaminostilbene by intraperitoneal injection and they were killed at intervals of ½ hour, 1 hour, 2 hours, 6 hours and 24 hours after the time of injection ; they will be referred to as $A_{\frac{1}{2}}$, A_1, A_2, A_6 and A_{24} respectively.

(b) *Intravenous.*—Some animals were anaesthetized by injecting intraperitoneally 1 ml. of a 2 per cent solution of Numal Roche. Because a good airway is essential with this anaesthetic, a tracheotomy was performed. An incision was made in the trachea and a plastic cannula tied in position. The portal vein was exposed and a plastic cannula was ligatured into position ; the 4-dimethylaminostilbene (1 ml. of the 1 per cent solution in arachis oil) was injected into the cannula, and the animal was killed 4 minutes later.

4. *Technique of section cutting*

A sample of each of three lobes of the liver was taken and frozen immediately at between −40° and −70° C. Sections were cut at 8 μ on a freezing cryostat microtome at about −20° C. Details of this procedure, which is similar to that used by Coons, Leduc and Kaplan (1951) will be discussed elsewhere.

The sections were dried over phosphorus pentoxide in an evacuated dessicator for 1 hour at −30° C. and then for 1 day at 0° C., except when used in studies on succinic dehydrogenase activity, where they were stained immediately.

5. *Histochemical methods*

(a) *Fluorescence microscopy.*—Freshly prepared frozen sections were examined by fluorescence microscopy for the presence of the fluorescent carcinogen. The apparatus was as described by Baldwin *et al.* (1960) ; the exciting light was mainly of 365 mμ wavelength.

In addition, the sections were immersed in a saturated caffeine solution to which had been added, drop by drop, a solution of about 10 mg. of 4-dimethylaminostilbene in acetone until precipitation occurred. The sections were then examined by fluorescence microscopy.

(b) *Method for demonstrating succinic dehydrogenase.*—Freshly prepared frozen sections were incubated at 37° C. in a 0·1 per cent solution of neotetrazolium in a 0·05 M phosphate buffer (Sorensen's) at pH 7·8, to which was added sodium succinate at a concentration of 0·05 M. Nitrogen was bubbled through the solution before use. The control incubation solution lacked the succinate. The

method, on frozen sections, gave semi-quantitative results, depending on the time required to yield appreciable colour, or to produce the same amount of colour as a standard. After they were stained, the sections were mounted directly in Farrant's medium.

(c) *Janus Green.*—Freshly prepared frozen sections were mounted in a 0·01 per cent solution of Janus Green in 0·85 per cent sodium chloride.

(d) *Oil Red O.*—Freshly prepared frozen sections were fixed for 5 minutes in a 10 per cent solution of neutralized formalin (40 per cent Formaldehyde) containing 0·9 per cent sodium chloride. They were rinsed in distilled water, immersed in 60 per cent iso-propyl alcohol for 3 minutes and then in the Oil Red O solution (saturated Oil Red O in isopropyl alcohol 3 parts, distilled water 2 parts) for 10 minutes. They were rinsed in 60 per cent isopropyl alcohol and then in distilled water, counterstained with Harris' haematoxylin and mounted in Farrant's medium.

RESULTS

1. *Observations by fluorescence microscopy*

(a) *Normal and control livers.*—Normal and control liver sections exhibited a faint autofluorescence; the liver cells were very pale green with brighter green outlines and dark non-fluorescent nuclei.

(b) *Liver sections from injected animals.*—The carcinogen, dissolved in arachis oil, emitted a strong blue fluorescence under ultraviolet light—arachis oil itself was non-fluorescent.

Liver sections from animals treated with intraperitoneal injections of 4-dimethylaminostilbene showed no increase of fluorescence. In order to ensure that the carcinogen was reaching the liver, it was injected directly into the portal vein in some animals. When sections of the liver were examined, no characteristic fluorescence was seen.

(c) *"Staining" of normal and control liver sections by 4-dimethylaminostilbene.*—When normal and control liver sections were examined by fluorescence microscopy after "staining" with an aqueous suspension of 4-dimethylaminostilbene, the entire section exhibited the bright blue fluorescence of the carcinogen. This fluorescence faded uniformly and after 5 minutes the sections showed only normal autofluorescence.

(d) *"Staining" of liver sections from injected animals by 4-dimethylaminostilbene.*—Although sections of the livers of animals injected with the carcinogen did not show fluorescence due to the aminostilbene, they could be "stained" by immersion in an aqueous suspension of 4-dimethylaminostilbene; as with the control sections, this induced fluorescence faded within 5 minutes. In addition, "stained" sections from the animal killed half an hour after injection showed bright blue fluorescent droplets in the periportal cells, which retained the fluorescence for 15 minutes, that is, for 10 minutes after the generalized fluorescence had faded. It could be demonstrated by staining with Oil Red O that these were fat droplets.

(e) *To test whether fading of the fluorescence of 4-dimethylaminostilbene was due to quenching by cellular constituents.*—Normal liver sections, fixed by formalin, and then "stained" in an aqueous suspension of 4-dimethylaminostilbene, exhibited generalized bright blue fluorescence. On repeated examination by fluorescence microscopy, this fluorescence was constant and did not fade.

Despite this result, it was apparent that the fading of the fluorescence could be due to quenching by some unknown cellular constituent which is inactivated by formalin. However, in an animal which died under anaesthetic, and which was injected intravenously into the portal vein immediately after death, the fluorescence of the carcinogen was seen strongly throughout the sinusoids and also in the cytoplasm of the liver cells in frozen sections. Hence, where it might be expected that metabolic activity, particularly oxidation, had been stopped, no fading occurred.

2. *Histochemical estimation of succinic dehydrogenase activity*

Succinic dehydrogenase activity was estimated by the rate of appearance and intensity of red stained granules in the cells. In the livers of animals A_N and A_C, cytoplasmic granules were coloured red after half an hour's incubation, with very intense staining after 2 hours. In animal $A_{\frac{1}{2}}$ no stain was observed after half an hour and only faint coloration after 2 hours' incubation. The staining of animals A_1 and A_2 was stronger than $A_{\frac{1}{2}}$ but still weaker relative to A_N. The cytoplasmic granules in the liver cells of A_6 and A_{24}, however, stained as did the normal, that is the succinic dehydrogenase activity was recovered 6 hours after injection.

To assess the relative activity in $A_{\frac{1}{2}}$, sections of $A_{\frac{1}{2}}$ were incubated for 2 hours and the intensity of reaction seemed identical with that seen in sections of A_6 incubated for only 1 hour. Hence the succinic dehydrogenase activity in $A_{\frac{1}{2}}$ was reduced by approximately one half.

3. *Janus Green staining*

Since succinic dehydrogenase is typically a mitochondrial enzyme, it was advisable to see how far the changes in the activity of this enzyme could be correlated with morphological alteration. For this purpose, sections were stained with Janus Green. Mitochondria were seen in various forms; filaments, rods, granules and globules could be identified, all of which stained selectively with this dye.

Filamentous mitochondria were found only in the normal and control livers. After injection of carcinogen, the mitochondria were in the form of short rods and granules. Globular forms were also seen in some of the liver cells and persisted in the livers of the animals killed 6 and 24 hours after injection. In addition green staining droplets were seen in the cytoplasm of the periportal liver cells of the animal killed half an hour after injection. These droplets stained with Oil Red O, indicating that they consisted of fat. Fat droplets were also present in the periportal cells of the liver sections from the animal killed 1 hour after injection, and to a lesser extent in the animal killed 2 hours after injection, but there were no fat droplets in the sections from the animals killed 6 and 24 hours after injection.

DISCUSSION

1. *Fluorescence*

After intravenous or intraperitoneal injection of 4-dimethylaminostilbene, no added fluorescence can be seen in the liver cells. The blue fluorescence of 4-dimethylaminostilbene may be masked by the normal autofluorescence, or its fluorescence may be quenched, or the carcinogen may be rapidly metabolized

and the fluorescence destroyed. When a solution of 4-dimethylaminostilbene is viewed under ultraviolet light, the blue fluorescence is constant and does not fade, showing that it is not destroyed by ultraviolet light. Moreover, when sections are " stained " by a suspension of the carcinogen, blue fluorescence is visible and is distinct from the autofluorescence. Hence, if the fluorescence of the carcinogen were present, it would have been seen and therefore almost certainly was not masked by the autofluorescence.

Snapper *et al.* (1951) found that the fluorescence of stilbamidine was quenched by nucleic acids. That this is the explanation of the results reported in the present communication is unlikely for two reasons. Firstly, no fading occurred when a section, fixed by formalin, was " stained " by a suspension of 4-dimethylaminostilbene ; it is possible, however, that some unknown cellular constituent, responsible presumably for quenching, had been inactivated by formalin. Secondly, examination of a section, taken from an animal which had died under anaesthetic and which was injected intravenously into the portal vein immediately after death, showed brilliant fluorescence of the carcinogen which persisted and was not quenched ; in this case metabolic activity, particularly oxidation, had presumably been stopped and no fading of fluorescence was observed.

Thus it appears likely that the fading of the fluorescence that occurs after " staining " with the carcinogen, and the lack of fluorescence in the injected animals is due to rapid metabolism of the carcinogen to a non-fluorescent form. Where the " stain " is taken up preferentially by fat droplets in the liver cells, the fluorescence is destroyed less rapidly, fading after about 15 minutes, and this delay is probably due to the partial protection against metabolism afforded to the carcinogen by the fat droplets.

2. *Succinic dehydrogenase activity and Janus Green results*

After injection of 4-dimethylaminostilbene, the mitochondria lose their filamentous form and appear as rods and granules. Similarly, in the animal killed half an hour after injection, succinic dehydrogenase activity is much reduced. In the animals killed at longer intervals after injection, however, although the mitochondria still appear damaged morphologically, the enzyme activity has been restored to the normal level.

At first sight, these observations could be interpreted simply as follows : the fact that the mitochondria can be deformed grossly and yet stain well for the enzyme suggests that their morphological integrity is not essential for succinic dehydrogenase activity. Hence the reduction in the activity of this enzyme shortly after injection of the carcinogen implies a direct inhibition of the enzyme. Such an inhibition has been demonstrated by Elson (1952, 1958) for oxidized metabolites of the related carcinogen dimethylaminoazobenzene. If this suggestion is correct and if oxidized metabolites of 4-dimethylaminostilbene are present half an hour after injection, then it is not surprising that little or no fluorescence due to the intact molecule, has been observed in the liver cells.

The speed of metabolism of the 4-dimethylaminostilbene might appear excessively fast but such a rapid metabolism of the related dimethylaminoazobenzene has been demonstrated by Mueller and Miller (1948), who showed that demethylation began at 10 minutes and was almost complete at 30 minutes. It is obvious that the final proof that 4-dimethylaminostilbene is metabolized depends on biochemical investigations which are now being undertaken.

CONCLUSIONS

After a single injection of 4-dimethylaminostilbene, neither protein binding nor permanent depression in succinic dehydrogenase activity can be demonstrated in the liver cells. It seems clear, however, that the liver cells react sharply to the carcinogen. Further injections of the carcinogen may modify this initial reaction.

SUMMARY

1. The localization of the strongly fluorescent carcinogen 4-dimethylaminostilbene was studied by fluorescence microscopy in frozen sections of rat liver. No characteristic fluorescence was seen after intraperitoneal or intravenous injection of the stilbene. The fluorescence faded rapidly in frozen liver sections "stained" with 4-dimethylaminostilbene, but persisted in dead or fixed liver sections. These results appeared to indicate rapid metabolism of the carcinogen and there was no evidence that the whole molecule became bound in the liver cells.

2. Morphological damage to the mitochondria of the liver cells appeared soon after injection and persisted for at least 24 hours. There was concomitant depression of succinic dehydrogenase activity soon after injection, but this recovered to normal levels in a few hours. It appeared likely that succinic dehydrogenase was directly inhibited, probably by metabolites of the carcinogen.

REFERENCES

Baldwin, R. W., Beswick, J., Chayen, J. and Cunningham, G. J.—(1960) *Acta Un. int. Cancr.*, **16**, 47.
Coons, A. H., Leduc, E. H. and Kaplan, N. H.—(1951) *J. exp. Med.*, **93**, 173.
Elson, L. A.—(1952) *Brit. J. Cancer*, **6**, 393.—(1958) *Brit. med. Bull.*, **14**, 161.
Idem and Hoch-Ligetti, C.—(1945) *Biochem. J.*, **40**, 380.
Miller, E. C. and Miller, J. A.—(1955) *J. nat. Cancer Inst.*, **15**, 1571.
Mueller, G. C. and Miller, J. A.—(1948) *J. biol. Chem.*, **176**, 535.
Snapper, I., Schneid, B., Lieben, F., Gerber, J. and Greenspan, E.—(1951) *J. Lab. clin. Med.*, **37**, 562.

A HISTOCHEMICAL STUDY OF THE EARLY STAGES OF CARCINOGENESIS IN RAT LIVER : CHANGES IN CELLULAR LIPIDS AND MITOCHONDRIA

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CHOLANGIOMATA occur in a high proportion of rats that have been injected intraperitoneally with 4-dimethylaminostilbene, particularly if they are fed on a diet containing a " low concentration " of protein (Elson, 1952). This carcinogen is particularly useful for investigations of the mechanism of tumour induction since it emits a blue fluorescence when activated by ultra violet light, thus allowing its intracellular distribution to be observed by fluorescence microscopy. Such studies showed, however, that the fluorescence due to the presence of 4-dimethylaminostilbene in cells of the rat liver faded rapidly (Bitensky, Baldwin and Chayen, 1960). This might have been due either to metabolic changes in the molecule or to the speedy excretion of the carcinogen. The following is a report of histochemical investigations made to elucidate the immediate response of the liver to the carcinogen.

MATERIALS AND METHODS

Materials and methods are as described in the preceding paper (Bitensky, Baldwin and Chayen, 1960).

Histochemical methods

(a) *Acid haematein method* (Baker, 1946).—Freshly prepared frozen sections were fixed in the dichromate mordant at 22° C. for one day and then at 60° C. for one day. They were rinsed in distilled water, immersed in the solution containing acid haematein for 5 hours at 37° C. and then in the borax-ferricyanide differentiating solution for 18 hours at 37° C. The sections were rinsed, dehydrated and mounted in Canada Balsam.

A positive reaction with the acid haematein method is thought to indicate the presence of phospholipid (Baker, 1946 ; Chayen, Gahan and La Cour, 1959). It was difficult to record accurately the great variation of intensity of the acid haematein reaction. This was overcome by the use of Kodak " Microdak " panchromatic 35 mm. film which was found to give a sufficiently linear response over the wide range of light intensities encountered. This film requires special handling and processing, and reference should be made to the manufacturer's instructions. The yellowish non-specific background produced by the acid haematein method was suppressed by the use of a magenta colour filter (Wratten No. 35). Thus, to produce accurate representation of the various intensities of

reaction with this staining technique, each slide was photographed in magenta light with a standard exposure and under standard optical conditions. All the subsequent photographic procedures were equally standardized. The "Micro-dak" film ensured that all the exposures gave a proportionate photographic response.

(b) *Extraction by methanol-chloroform.*—Freshly prepared frozen sections were immersed in 1 : 2 (v/v) methanol-chloroform for 4 hours at 22° C. They were then treated with the acid haematein method as described above.

(c) *Prolonged acid haematein method.*—To demonstrate the more tightly bound phospholipids, freshly prepared frozen sections were fixed in the dichromate mordant for three days at 60° C. (see "Discussion"). They were then treated by the acid haematein procedure as described above.

Sections were stained with Oil Red O and Janus Green as already described (Bitensky, Baldwin and Chayen, 1960).

RESULTS

1. *Oil Red O*

Oil Red O stained neither normal nor control liver sections, nor did it stain liver sections taken from the animal injected with arachis oil alone. In the liver of the animal injected intravenously and killed after 4 minutes, some cells distributed at random contained stained fat droplets. The liver sections of the animal killed half an hour after intraperitoneal injection, however, showed a marked periportal fatty change with a small centrilobular region of liver cells free from fat (Fig. 1). In the rats killed between 1 and 6 hours after injection, the fatty change gradually diminished in extent (Fig. 2 and 3) and the liver of the animal killed 24 hours after injection contained almost no fat droplets, although small flecks which stained with Oil Red O were seen.

2. *Mitochondria*

Mitochondria were identified in this study by two techniques, namely, by staining with Janus Green and with the acid haematein reaction. Both these methods stained filaments, rods, granules and globules.

Filamentous mitochondria were found only in the normal and control livers (Fig. 7). After injection of the carcinogen, the mitochondria were in the form of short rods and granules; globular forms were also seen in some of the liver cells, and persisted in the liver of the animal killed 24 hours after injection (Fig. 8).

3. *Acid haematein staining of the cytoplasm*

In the liver of the animal fed on a complete diet, the cytoplasm, excluding the mitochondria, stained only a very weak brown, in contrast to the intense reaction found in the cells of the control rat fed on the low protein diet. In both the normal and control, some cells, scattered at random in the tissues, stained more strongly than the rest. The effect of injecting the carcinogen into the animals on the low protein diet was seen most strikingly in the changes in the acid haematein (phospholipid) staining. Thus the cytoplasm in the control rats stained intensely brown; in all the animals killed up to 6 hours after injection, the acid haematein method gave a very reduced blue-grey colour (Fig. 4, 5, 6).

In the livers of the injected animals there were some cells that stained more darkly, but unlike the random arrangement of such cells in the control, they showed a definite centrilobular distribution. Twenty-four hours after injection, recovery seemed to have begun in that the cells stained brown, although not as strongly as did the controls, and the distribution of the darker-staining cells had reverted to random.

4. *Acid haematein staining after methanol-chloroform*

In the normal and control livers, the vessels and sinusoids stained blue, as did the cytoplasm of a few of the liver cells. The remaining liver cells were coloured brown and the mitochondria were still visible. After injection of the carcinogen, a similar blue reaction was seen in the vessels and sinusoids of the liver, but in the animals killed up to 24 hours after injection, none of the liver cells stained blue. The periportal cells remained almost unstained, while in the centrilobular cells, the cytoplasm was coloured pale grey. In the liver of the rat killed 24 hours after injection, a few of the liver cells showed the blue staining and the cytoplasm of the remainder appeared brown in colour.

5. *Prolonged acid haematein method*

After treatment for three days in the hot dichromate, the cytoplasm of the liver cells in the normal animal stained dark brown, while the mitochondria were blue-black. The intensity of the stain was increased in the control livers, however, and was greater still in the liver from the animal killed 24 hours after injection. A similar enhanced staining was also seen in the centrilobular areas of the liver from the animals killed up to 6 hours after injection, but the periportal cells remained unstained and appeared empty.

DISCUSSION

1. *Results with Oil Red O and with the acid haematein procedure*

Oil Red O is simply a colorant of fats, that is, it colours regions containing a high concentration of lipoidal matter, but like many " fat stains " it does not demonstrate highly dispersed or hydrophilic lipids (Berg, 1951 ; Chayen *et al.*, 1959). The acid haematein reaction, on the other hand, is considered to show the presence of phospholipids. Although Baker (1946) believed that it needed to be controlled by his pyridine extraction test, this has now been found to be unnecessary and even, occasionally, misleading (Chayen *et al.*, 1959). This was due, in part, to the effect of heat used in the extraction test, rendering certain bound phospholipids more available for the acid haematein reaction. Consequently, in the present study tests for such bound phospholipids were made by prolonged treatment with hot dichromate, so that the dichromate mordanted the phospholipids immediately the heat freed them (Chayen, unpublished data). Hence, a positive reaction with the acid haematein method will be considered as indicating the presence of phospholipids.

In these studies, two types of phospholipid could be distinguished, namely, that present in the mitochondria and that dispersed in the cytoplasm. Even though the morphology of the mitochondria was much altered, the intensity of their staining with the acid haematein method did not change appreciably with

TABLE I.—*Histochemical Results in Normal and Control Livers and in Livers of Animals Injected with 4-Dimethylaminostilbene*

Animal	Oil Red O		Acid haematein procedure			Janus Green mitochondria	Acid haematein procedure after methanol-chloroform extraction		Acid haematein procedure after prolonged mordant
	Droplets	Distribution	Background	Distribution	Mitochondria		Background	Mitochondria	
Normal	−	−	++	Random	Filaments	Filaments	+ Smeary blue	+	+
Control	−	−	+++	,,	,,	,,	Ditto	+	++
Arachis oil injection	−	−	++	,,	,,	,,	,,	+	+
Intravenous injection of 4-DAS*	+	Random	+	Centrilobular	Rods and granules	Rods, granules and globules	None	+	+++ Centrilobular
½ hr. after injection of 4-DAS	++	Periportal	±	,,	Rods, granules and globules	Ditto	,,	+	Ditto
1 hr. after injection of 4-DAS	+	,,	±	,,	Granules and globules	Granules and globules	,,	+	,,
2 hr. after injection of 4-DAS	+	,,	+	,,	Rods, granules and globules	Rods, granules and globules	,,	+	,,
6 hr. after injection of 4-DAS	±	,	+	Random	Ditto	Ditto	,,	+	,,
24 hr. after injection of 4-DAS	−	−	++	,,	,,	,,	+ Smeary blue	+	,,

* 4-DAS = 4-dimethylaminostilbene.

time after injection of the carcinogen and hence this type of phospholipid has probably not contributed to the changes seen in the cytoplasm.

The perplexing feature of the results (Table I) is the fact that within 4 minutes of intravenous injection, or half an hour of intraperitoneal injection, there was a striking apparent loss of cytoplasmic phospholipid with concomitant increase in free fat. It was shown that this was not due to deposition of arachis oil into the liver. Moreover, it was improbable that it was caused by two distinct mechanisms, one depositing fat in the liver and the other removing phospholipid, all acting within such a short time. This is almost ruled out of consideration, because no phospholipid was observed either leaving the liver cells, or in the bile canaliculi, so soon after injection. It is also unlikely, in the short time after injection, that the freed phospholipid was undergoing enzymic removal of its phosphate moiety and being deposited as fatty droplets, but this possibility cannot be discarded without more biochemical, and preferably isotopic, studies.

It seems more likely that the carcinogen caused a change of phase, altering a "fat-in-water" into a "water-in-fat" emulsion. It is envisaged, therefore, that in the normal liver cells, the cytoplasmic phospholipid is tightly bound and occurs with its hydrophobic groups away from, and its hydrophilic groups outwards to, the watery cell sap. In the animals fed plentifully with protein, these hydrophilic groups may be protected from the usual acid haematein procedure by protein; in the rats fed on a diet deficient in protein, this protection is not present and hence the phospholipids are more available to the acid haematein reaction. The carcinogen seems to strip the phospholipids from the protein (see below) and to leave the phospholipid in a reversed condition, namely with its fatty moieties outermost, and run together in droplets which are gross enough to colour with Oil Red O. In consequence, the hydrophilic (phosphate or rather phosphatidyl choline, etc.) groups become turned inwards and therefore are no longer available to the acid haematein reaction.

This is a complex hypothesis, but one that can be tested in the biochemical studies that are now in progress.

EXPLANATION OF PLATES

FIG. 1.—Rat liver ½ hour after injection of 4-DAS; phase contrast illumination, ×500; to show droplets of free fat, which appear as white spheres (cf. Fig. 4 for phospholipid content).

FIG. 2.—Rat liver 2 hours after injection; phase contrast illumination, ×500; the fat droplets already are less obvious than in Fig. 1 (cf. Fig. 5).

FIG. 3.—Rat liver 6 hours after injection; phase contrast illumination, ×500; there is practically no free fat and recovery is almost complete (cf. Fig. 6 for similar recovery of phospholipid content).

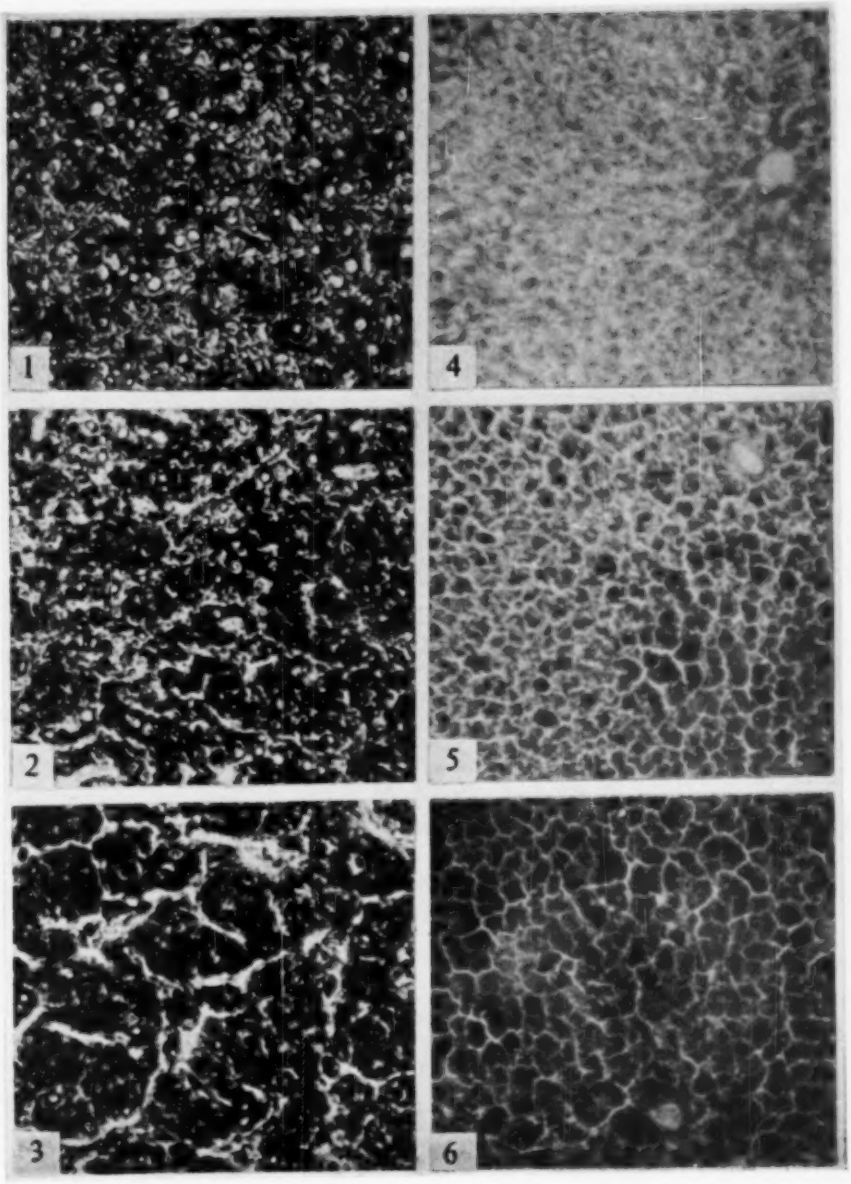
FIG. 4.—Rat liver ½ hour after injection; acid haematein method, ×500; note the low content of phospholipid, i.e. the paleness of the stain, in contrast to the high fat content of the similar section in Fig. 1.

FIG. 5.—Rat liver 2 hours after injection; acid haematein method, ×500.

FIG. 6.—Rat liver 6 hours after injection; acid haematein method, ×500; the phospholipid content is at least half-recovered, as shown by the darker staining of the cells, while the free fat content is almost completely suppressed (Fig. 3).

FIG. 7.—Normal rat liver cells with filamentous mitochondria; acid haematin method, ×1680.

FIG. 8.—Damaged mitochondria in rat liver cells ½ hour after injection of 4-DAS; acid haematein method, ×1680.



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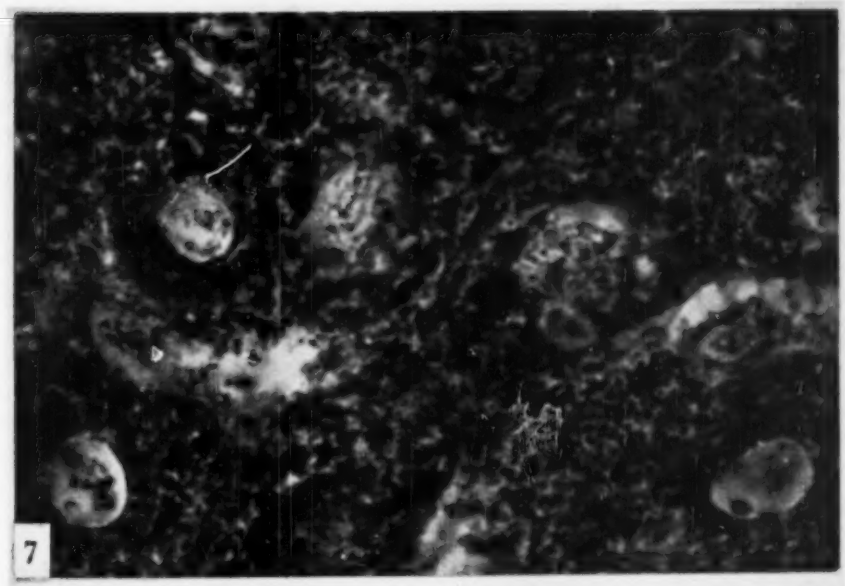

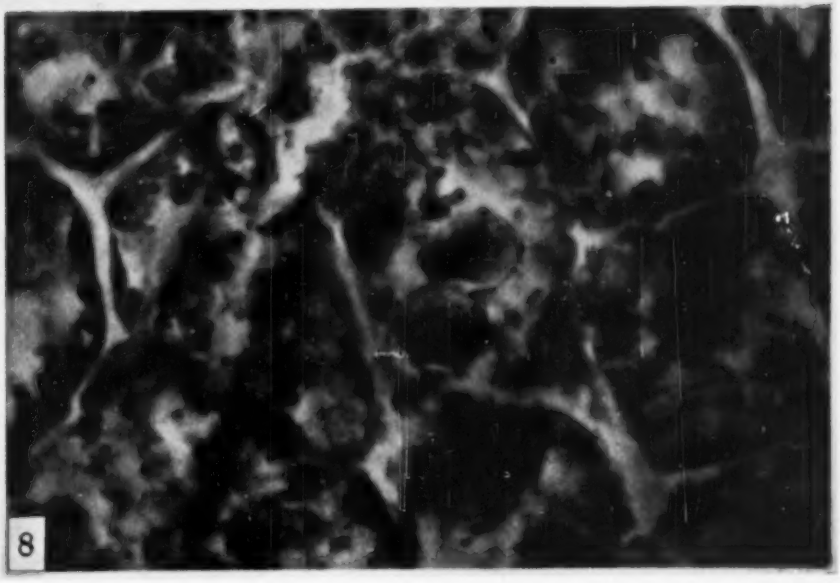

Bitensky, Baldwin and Chayen.

2. *Effect of methanol-chloroform*

When liver sections are subjected to extraction by a mixture of methanol and chloroform, the former tends to break lipid-protein bonds. If the action is complete, phospholipid is freed and extracted from the section. If, however, the liberation is incomplete, the previously bound phospholipid becomes available for staining by the acid haematein reaction.

In the normal and control livers, which had been treated with methanol-chloroform, serum lipids were partially liberated from protein and so stained blue within the blood vessels ; this effect was also seen in a few of the liver cells. No loss of staining occurred and therefore no phospholipid was freed sufficiently to be extracted.

3. *Effect of 4-dimethylaminostilbene on lipoprotein binding*

The almost complete removal of phospholipid by methanol-chloroform acting on sections of the liver from animals killed up to 6 hours after injection, suggests that the lipids were less tightly bound than in the control cells. That this is a specific effect on the cytoplasmic phospholipids was demonstrated by the fact that the serum phospholipids were unaffected and behaved like those of the control animals. Twenty-four hours after injection, the binding seemed to have reverted to the type found in the control.

It cannot be claimed that the results obtained after the prolonged treatment with dichromate mordant (3 days at 60° C.) demonstrate the total phospholipid content of these cells, but they give some indication of the more closely bound as well as the free phospholipids. It seems likely therefore that there is very much less staining for phospholipid in most of the cells up to 6 hours after injection than there is in the control. The exception to this is the cells with centrilobular distribution, which appear to contain even more phospholipid than do the control cells, suggesting that the carcinogen has accentuated the unmasking effect of the hot mordant. This view seems more likely than that there is a real increase in the lipid content, because of the speed with which the effect is produced.

4. *Mitochondria*

Filamentous mitochondria were seen in the liver cells of the normal and control animals. After injection of 4-dimethylaminostilbene, however, the mitochondria appeared as granules and short rods, but globular forms were also present especially after 6 and 24 hours. The damage to the mitochondria in the animals treated with carcinogen appears to be a direct effect of 4-dimethylaminostilbene.

5. *The general effect of the carcinogen on liver cells*

It is clear, therefore, that although the carcinogen cannot be localized within the liver by means of its fluorescence, nor by any histological abnormality so soon after injection, its presence can be inferred from the histochemical and cytological changes it causes. These include the breakdown of mitochondria and the considerable increase in free lipid, correlated with the apparent loss of phospholipid. The latter change together with the apparent increase in total phospholipid in the exceptionally staining centrilobular cells, suggest that the carcinogen or its metabolite, has freed the lipid-protein binding and caused some type of phase inversion, so protecting the freed phospholipids within droplets of fat.

SUMMARY

The immediate response of rat liver to the carcinogen, 4-dimethylaminostilbene, was studied by histochemical methods on frozen sections. There was a considerable increase in free lipid, correlated with an apparent loss of phospholipid in some of the liver cells soon after injection ; recovery occurred gradually within twenty-four hours. Other liver cells showed an apparent increase in total phospholipid content. These results suggested that the carcinogen, or a metabolite, had freed the bonds between lipids and proteins, and caused some type of phase inversion. In addition, morphological damage to the mitochondria of the liver cells was confirmed.

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REFERENCES

Baker, J. R.—(1946) *Quart. J. micr. Sci.*, **87**, 441.
Berg, N. O.—(1951) *Acta path. microbiol. scand.*, Supp. 90.
Bitensky, L., Baldwin, R. W. and Chayen, J.—(1960) *Brit. J. Cancer*, **14**, 690.
Chayen, J., Gahan, P. B. and La Cour, L. F.—(1959) *Quart. J. micr. Sci.*, **100**, 325.
Elson, L. A.—(1952) *Brit. J. Cancer*, **6**, 393.

ENVIRONMENTAL FREE RADICALS

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In work related to the causation of human lung cancer, some investigators, appreciating the proposed special cancer environment of the cigarette smoker, seek to support this proposition by discovery of new carcinogenic agents or adjuvant factors in cigarette smoke, to which non-smokers are not especially exposed. Thus smoke phenols (Roe, Salaman and Cohen, 1959) and bases (Wynder and Wright, 1957) have been cited as co-factors in what would essentially be carcinogenesis by smoke polycyclic hydrocarbons. The discovery of appreciable quantities of free radicals in cigarette smoke (Lyons, Gibson and Ingram, 1958) led to the proposal of their possible involvement in the carcinogenic process. This followed from various suggestions in the literature on the possible pivotal role of free radical forms in cancer production by a variety of agents (for reference see Bamford and Jenkins, 1960). A more speculative aspect, perhaps, of the free radical hypothesis is the possible sensitization of cells to the deleterious action of carcinogenic agents by free radicals through their paramagnetism, an idea related to that of Howard, Hawes and Gray (1959) who are seeking to establish whether the well-known radiosensitizing action of oxygen and nitric oxide is due to the paramagnetic property of these agents.

For present purposes it was decided to attempt an estimation of the relative amounts of free radicals to which cigarette smokers and non-smokers are liable to be exposed, and to investigate any qualitative differences among such radicals which might have a bearing on their possible biological action. As regards the latter point, it seemed desirable to have an estimate of their size and stability. For these ends, the following experimental work was undertaken.

EXPERIMENTAL

Free Radicals in general atmospheric pollution

Suspected sources of general atmospheric free radicals examined were: domestic chimney smoke, vehicular exhausts and cigarette side-stream smoke.

Samples of particulate exhaust products from mechanically sound petrol and diesel enges were collected in glass containers which were kept cool during the sampling. The diesel soot samples were collected at speeds of 2080, 1400 and 800 r.p.m., at 10 per cent overload from vehicle engines on a test bed. (Negligible quantities of soot were produced at reduced loads.) The petrol engine soots were collected during acceleration-deceleration and idling periods from privately owned cars. All the exhaust condensates were filtered and dried over phosphorus pentoxide. These samples were tested for the presence of free

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radicals by the electron spin resonance (e.s.r.) method, as were samples of domestic chimney soot and general atmospheric soot—the latter collected over a two-month period in autumn in a ventilator shaft at a site in central Glasgow.

In view of the well-known conditional carcinogenicity of soot-borne carcinogens (Steiner, 1954 ; Nau, Neal and Stembridge, 1958 ; Von Haam, Titus, Caplan and Shinowora, 1958) it was of interest to know the proportion of soot radicals likely to be available to the cells of the respiratory tract—as far as this can be known by extraction with organic solvents. Consequently, the radical levels of the soots were re-determined following the extraction of aliquots with hexane, benzene and acetone. The results were as follows :

(1) The domestic chimney soots contain 5×10^{18} free electrons per gram of soot. This concentration was not affected by the extracting solvents, hexane, benzene and acetone, which removed 6·6, 27·5 and 41·0 per cent by weight of material, respectively.

(2) The diesel soots contained $1{\cdot}8 \times 10^{19}$ free electrons per gram, irrespective of running condition. This level was also unaffected by the extractions, which resulted in a loss by weight of 2, 6 and 7 per cent.

(3) Both petrol exhaust samples exhibited an extremely large broad resonance signal (of the order of 1000 gauss) which was unaffected by any of the solvents used.

(4) The ventilator soot also exhibited a large broad resonance.

In the case of the petrol and ventilator soots it has been suggested (B. T. Allen, personal communication) that the origin of the large broad resonance may lie in the presence of lead and iron in the samples. The free electron concentrations were not available at the time of writing. The domestic soot samples exhibit normal free radical absorption similar to carbon blacks, while the diesel soot absorption is broader, although still centred around $g = 2$. The broader absorption signal of the diesel soot relative to the domestic soot is related to its formation at a higher temperature, and is due to the interaction of the free electrons with electrons in the conduction bands (Austen, Ingram and Tapley, 1958).

Cigarette side-stream smoke was found to contain approximately 5×10^{14} free electrons per gram. Main-stream smoke had a " stable " radical concentration about twice as high.

Free radicals in cigarette main-stream smoke

Cigarette main-stream smoke, condensed at liquid oxygen temperatures showed the presence of 6×10^{15} free electrons per gram. On warming to room temperature or above, or trapping the smoke in benzene at room temperature a residual concentration of about 10^{15} was found. A majority of these were found to be light sensitive, as the following experiments indicate.

Drückrey and Schmähl (1955) showed that the fluorescence intensity of freshly prepared benzene solutions of cigarette smoke decreased on exposure to light. Johnston (1957) showed that the labile fluorescent components were products of combustion of the tobacco ; that similar labile material could be produced by combusting other vegetable matter ; that the percentage of the total fluorescent material which was labile varied for the various material combusted.

In the present investigation it was found that the reactivity of fresh benzene solutions of cigarette smoke with the stable free radical scavenger $\alpha\alpha'$-diphenyl-

β-picrylhydrazyl (DPPH) decreased on exposing the former to light. This decrease was found to occur even when the tobacco was extracted with acid, alkali and organic solvents prior to smoking. DPPH-reacting polar constituents of the smoke were found not to be light sensitive. It was decided to test whether the decrease in fluorescence intensity and in DPPH-activity were related phenomena and if so, to attempt an explanation.

DPPH-reacting polar reducing substances were extracted from the cigarettes prior to smoking by refluxing with dilute alkali, acid and water. After conditioning the tobacco to about a 10 per cent water content the tobacco was smoked in cigarette form. The smoke from one such cigarette in 250 ml. of benzene afforded a convenient solution for both fluorescence and DPPH-activity measurements.

Solutions were irradiated at a distance of 25 cm. with an unfiltered high pressure 125 W mercury vapour lamp, giving a band spectrum, and samples withdrawn at hourly intervals for measurement. Fluorescence measurements were made on a Uvispek spectrophotometer with a H730 fluorescence attachment, which afforded excitation at 365 mμ. An 0·1 mg. per cent quinine sulphate in 0·1 N . H_2SO_4 standard was used. DPPH-activity was estimated from observing the rate of decrease of the compound's 520 mμ absorption maximum following the addition of 0·1 ml. of a 10^{-2} M solution to 10 ml. of the smoke solution. The percentage decrease in both cases was correlated with concentration by reference to dilution curves for the same solution.

A remarkably close correspondence between the decrease in fluorescence intensity and DPPH-activity was found. This is shown in Fig. 1.

An analysis of the curves shows them to be discontinuous at two points, the four- and six-hour irradiation points. A decrease of about 60 per cent in the concentration of labile components occurred in 24 hours, the major portion (50 per cent) occurring in the first seven hours of irradiation. The reaction had first order kinetics, and three different rate constants revealed the presence of three different components. Electron spin resonance measurements of a smoke solution irradiated under the same conditions for two and six hours showed that decreases in free radical concentration of 26 and 40 per cent respectively had occurred.

The light sensitive components of cigarette smoke appeared therefore to be free radicals, a light titration of which revealed the presence of at least three species of different stability.

In order to obtain some estimate of the size of such radicals, a concentrated benzene solution of smoke was added to fresh alumina (Spence, 100–200 mesh), and the solvent allowed to evaporate off in the air. The resulting alumina with adsorbed tar was filled into a column. Care was taken throughout to shield the tar from diffuse daylight. The column was washed with *n*-hexane until no further material was being eluted, and the same procedure carried out with benzene and acetone consecutively. The solvents were evaporated from the three eluates and the free radical content of the resulting tars estimated by e.s.r. The results are shown in Table I.

No radicals were detected in the hexane soluble fraction while the subsequent benzene and acetone eluates contained 35 per cent and 50 per cent of the radicals present in the original tar. Thus about 85 per cent of the radicals could be desorbed from the alumina with a facility which increased with the polarity of the

TABLE I.—*Chromatographic Behaviour of Cigarette Smoke Free Radicals*

Eluate	*% Eluted*
n-Hexane	0
Benzene	35
Acetone	0

solvent. The experiment indicates that some free electrons are trapped in individual aromatic structures containing as little as, perhaps, 4 or 5 condensed nuclei. Such structures, it is thought, could readily gain access to the cell.

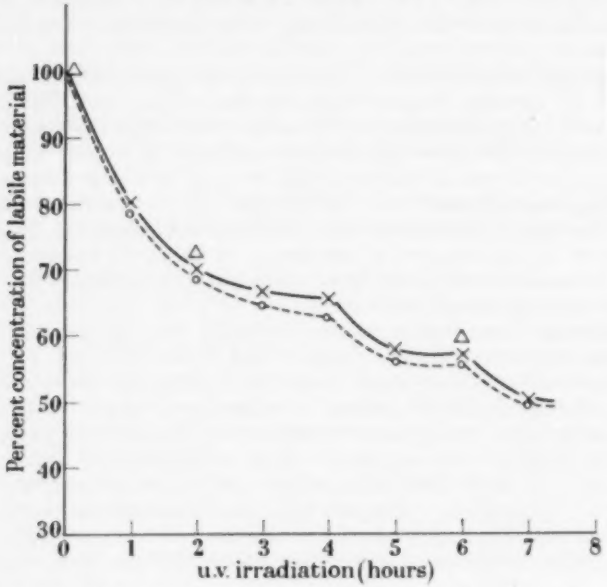

FIG. 1.—Light titration of photo-labile material of cigarette smoke.
×———× DPPH reaction.
○- - - -○ Fluorescence.
△ △ e.s.r. measure of free radicals.

DISCUSSION

The mechanism of free radical production in the carbonization of various organic materials proposed by Austen, Ingram and Tapley (1958) can, it is thought, be advanced to explain radical production from liquid fuels (in the present case, diesel and petrol fuels) as well as domestic fuels. These authors state that radical concentration grows when the carbon atoms begin to cluster in condensed ring systems, suggesting that the essential mechanism in the trapping and stabilization of the unpaired electrons is the existence of ring clusters possessing a high degree of resonance energy available for stabilization. It was assumed that the radicals were formed by breakage of bonds around the edge of carbon clusters, but they state, the possibility of their arising from defects in ring packing is

conceivable—five and seven membered rings producing internal trivalent carbon atoms.

The present work shows that the radicals, in the diesel soots and domestic chimney soot at least, are similar to those in carbon blacks in being extremely stable and insoluble. They possess a very similar absorption line.

Their common property, i.e. their radical content, is unimpaired by extraction with organic solvents. But since extracted carbon blacks are non-carcinogenic in contradistinction to the unextracted materials which are frequently active, it is anticipated that the type of free radical contained in these soots is non-carcinogenic.

In a previous experiment (Lyons, Gibson and Ingram, 1958) a benzene extract of general atmospheric soot was found to have a radical activity of approximately 10^{17} free electrons per gram. This was an over-estimate since the extract contained transition elements. The extract amounted to 20 per cent of the whole soot by weight. On the basis of Waller's figures (1952) about 1·43 g. of soot is inspired by the " standard man " per annum. Accepting the concentration 10^{17} as a maximum, and assuming that little arrest of the soot particles occurs and that the full radical load is available to the cells, an annual exposure to approximately 29×10^{15} free electrons occurs. A smoker of 30 cigarettes per day can have a yearly exposure of about 1970×10^{15} free electrons (on the basis of the smoker receiving 30 mg. of (dry) smoke per cigarette), i.e. about 68 times the amount received by the non-smoker. The radicals in the benzene extract of the atmospheric soot were not light-sensitive (in so far as this could be ascertained from reaction with DPPH), i.e. were of a higher order of stability.

Calculations based on such comparatively stable atmospheric free radicals can give a false picture however, since it is known that a profusion of active short-lived free radicals are present in polluted urban and industrial atmospheres. They have their origin in solar-initiated chemical events involving aliphatic hydrocarbons emitted into the atmosphere incidental to their use as liquid fuels. Such chemical events include the direct photolysis of aliphatic aldehydes and ketones and the reaction of photochemically produced ozone with olefins. Some degree of stabilization of these extremely reactive radicals occurs it is thought, by reaction with nitric oxide to form a complex (Saltzman, 1958) and perhaps by reaction with certain aromatic polycyclic hydrocarbons (authors' unpublished results). The total concentration of these free radicals in urban air may reach a level, 1 to 10 per cent of the molecular contaminants or alternatively, a steady state partial pressure of free radicals of 0·3 to 3·0 parts per hundred million (Johnston, 1956). It may well be, that this portion of the total atmospheric pool of free radicals carries greatest potential lung cancer hazard for humans. Further facts are required. Meanwhile, the smoker-non-smoker balance sheet remains incomplete.

SUMMARY

Diesel and petrol engine soots, domestic chimney soot, and a sample of general atmospheric soot were tested for the presence of free radicals using the method of electron spin resonance. Concentration levels of $1{\cdot}8 \times 10^{19}$ free electrons per gram and 5×10^{18} f.e/g. were obtained for diesel soots and chimney soot respectively. Due to the complicating effect of transition elements, the radical concentration of the petrol and atmospheric soots was not available at the time

of writing. It was shown that in the case of the diesel, petrol and chimney soots, the radicals were extremely stable and insoluble. It seems unlikely that they play any part in carcinogenesis.

The majority of the radicals trapped at room temperature in cigarette " tars ", in contradistinction to those of the soots, were found to be light sensitive, of a lower order of stability, and of a size range which would permit their finding access to the cell.

Other active free radicals, produced in polluted urban and industrial atmospheres by the action of sunlight on aliphatic material released into the air as a result of the incomplete combustion of liquid fuels, are briefly discussed, and their possible hazard for man indicated.

The authors are extremely grateful to Mr. B. T. Allen, of Professor Ingram's Department, University College of North Staffordshire, for the e.s.r. data.

REFERENCES

Austen, D. E. G., Ingram, D. J. E. and Tapley, J. G.—(1958) *Trans. Faraday Soc.*, **54**, 400.

Bamford, C. H. and Jenkins, A. D.—(1960) ' Formation and Trapping of Free Radicals ', p. 468. Edited by Bass and Brioda, New York and London (Academic Press).

Drückery, H. and Schmähl, D.—(1955) *Science*, **122**, 421.

Howard, A., Hawes, C. and Gray, L. H.—(1959) *Rep. Brit. Emp. Cancer Campgn*, **37**, 200.

Johnston, H. S.—(1956) *Industr. Engng Chem.* (*Industr.*), **48**, 1488.

Johnston, H.—(1957) *Nature*, **180**, 1350.

Lyons, M. J., Gibson, J. F. and Ingram, D. J. E.—(1958) *Ibid.*, **181**, 1003.

Nau, C. A., Neal, J. and Stenbridge, V.—(1958) *A.M.A. Arch. Ind. Health*, **11**, 21.

Roe, F. J. C., Salaman, M. H. and Cohen, J.—(1959) *Brit. J. Cancer*, **13**, 623.

Saltzman, B. E.—(1958) *Ind. Eng. Chem.*, **50**, 677.

Steiner, P. E.—(1954) *Cancer Res.*, **14**, 103.

Von Haam, E., Titus, H. L., Caplan, I. and Shinowora, G. Y.—(1958) *Proc. Soc. Exptl. Biol.*, **98**, 95.

Waller, R. E.—(1952) *Brit. J. Cancer*, **6**, 8.

Wynder, E. L. and Wright, G.—(1957) *Cancer*, **10**, 255.

THE MYELOMA GLOBULINS

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In 1935 Mcfarlane demonstrated, in the sera of a patient suffering from myelomatosis, a protein having an unusual sedimentation constant. It has been shown that these sedimentation constants vary according to the protein concentration at which they are estimated (Neurath and Bailey, 1953). If there is not gross asymmetry of the molecule examined, and this is the case for most serum proteins, the observed sedimentation is a linear function of concentration, and the sedimentation constant obtained by extrapolation to zero concentration is related to the observed sedimentation constant by the simple equation :—

$$S^{\circ}_{20} = S_{20} + kc$$

S_{20} being the observed sedimentation constant ;
S°_{20} the sedimentation constant extrapolated to zero ;
c the concentration of protein in grams per 100 ml. at which the observation was made.

Koenig and Pederson (1950) have obtained K values of 0·65 and 0·25 for fibrinogen and gamma globulin respectively. While Taylor (1952) obtained a K value of 0·235 for albumin. It will be seen that fibrinogen, the molecule with the highest degree of asymmetry, has the highest K value.

McConnell and Martin (1958), in the course of a physicochemical study of globulins isolated from individual patients suffering from myelomatosis, produced data on the relation of sedimentation to the concentration of the isolated products. Studies on isolated globulins have continued in this laboratory. Fig. 1 shows some of this accumulated data in condensed form.

When the K values of these proteins are plotted against their electrophoretic mobility, the appearance of the graphical results (Fig. 2) indicated that increasing asymmetry is associated with increasing negative net charge. These demonstrable physicochemical changes point to overall changes in the configuration of the globulin molecule, but they do not point to precisely defined groups of globulins so much as a spectrum of molecules with a gradation of change.

The careful studies of Wallenius, Trautmann, Kunkel and Franklin (1957) indicated that there were several components present in normal human serum with sedimentation coefficients other than those commonly observed in routine ultracentrifugal analysis.

Putnam and Udin (1953) suggested that the range and frequency of myeloma globulins in general correspond with those of normal gamma globulin. There has therefore always been the possibility that the relatively easily identified "abnormal" proteins observed in myelomatosis might represent the abnormal

production of proteins present, in trace amounts, in normal sera, a possibility considered by Slater, Ward and Kunkel (1955).

If, as some writers have maintained, these myeloma globulins are inherently different from normally produced gamma globulins, it is important to know to what extent and in what way they differ. To extend the study of these globulins it was thought that the immunoelectrophoretic technique of Williams and Grabar, modified for microanalysis by Scheidigger (1955), might yield useful information.

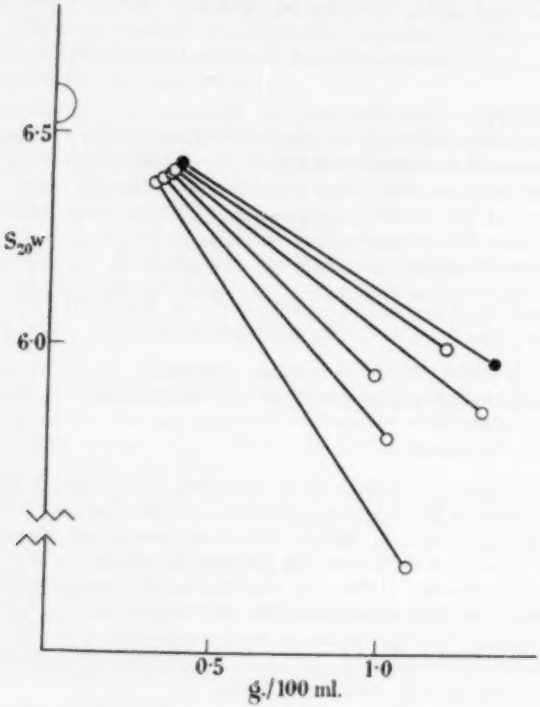

FIG. 1.—Sedimentation concentration ratio

●——● normal gamma globulin
○——○ myeloma gamma globulins

Each line represents the plot of the slope calculated from not less than five individual measurements.

MATERIAL AND METHODS

In these investigations fresh serum from fasting patients, in whom a diagnosis of myelomatosis had been established both on clinical and histological grounds, was used. Electrophoretic analyses were made in the Tiselius apparatus using 0·2 M phosphate buffer at pH 8·0 at 0°C. Ancillary electrophoretic investigations, when the material was insufficient for the classical technique, were made on paper by the method described by Franglen, Martin and Treherne (1955). Immuno-

electrophoretic analyses were made by the microtechnique described by Scheidigger (1955), and nitrogen estimations were done by the Micro-Kjeldahl method.

Preparation of antisera

Antisera were prepared in rabbits by two methods :

(1) Intramuscular injection of a suspension of an alumina precipitate of the antigenic protein, according to the method of Proom (1943) as used by Weitz (1952).

Six injections of 10 ml. of the suspension in a divided dose were given at ten day intervals. The titre started to rise after the second injection and had reached a satisfactory level after the fifth injection.

(2) Subcutaneous inoculation of the antigenic protein using Freunde's adjuvant.

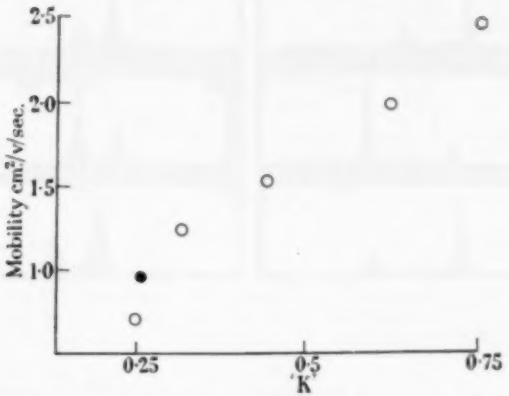

FIG. 2.—Electrophoretic mobility of normal and myeloma globulins plotted against K values derived from concentration sedimentation studies.

● normal globulin
○ myeloma globulins

The following antisera were used in these investigations :

(1) Antisera against the freshly drawn serum of two patients known to be suffering from myelomatosis, designated G and K, and showing anomalous protein patterns in the beta and gamma areas respectively.

(2) Antisera prepared against the G4 fraction of pooled normal sera containing 99 per cent gamma globulin.

Though the original G4 globulin used for the production of antibodies contained no fast moving globulins demonstrable by classical electrophoretic analysis, immunochemical analysis against normal sera suggested the presence of a trace of globulin having the mobility of alpha globulin (see Fig. 4, third column).

RESULTS

Table I shows the total serum proteins and the alpha, beta and gamma globulin content of the five sera subjected to immunochemical analysis. These

values were obtained by electrophoretic analysis in the classical Tiselius apparatus. Representative Schlieren patterns are shown in Fig. 3.

The immunoelectrophoretic analyses against the three specific antisera are shown in Fig. 4. Serial dilution studies of these proteins against the antisera did nothing more than emphasize the constant characteristics of the patterns obtained.

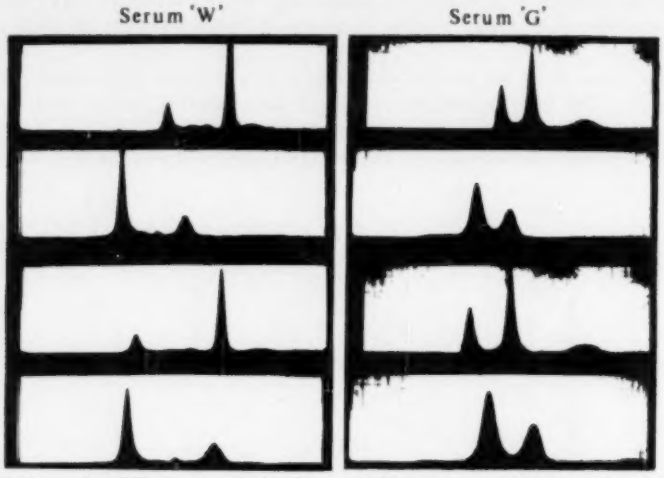

FIG. 3.—Photographs of Tiselius' analysis of serum "W" and serum "G" at 2½ and 3 hours. Analysis of a 2 g./100 ml. protein solution in 0·2 molar phosphate buffer at pH 8·0, at 0°C. and at a potential gradient of 6v/cm.

TABLE I.—*Serum Proteins and the Alpha, Beta and Gamma Content of the Five Sera Subjected to Immunochemical Analysis.*

Patient	Total protein g/100 ml.	Alb.	% Total of components (globulins)			
			alpha	beta	gamma$_1$	gamma$_2$
"W"	10	14	5	7	—	73
"R"	8·6	18	5	8	—	67
"T"	8·0	20	4	6	60	9
"G"	7·8	28	7	57	7	
"C"	9·9	21	8	63	7	

DISCUSSION

The physicochemical data referred to in the introduction suggest a progressive deviation in the structure of the myeloma from the normal globulins rather than a clear cut differentiation. Thirteen out of nineteen end group analyses of myeloma globulins listed by Putnam (1956) showed aspartyl or glutamyl or both to be present. These are the end groups commonly found in analysis of gamma globulin. The carbohydrate analysis of Müller-Eberhard and Kunkel (1956) and of McConnell and Martin (1958) showed variations in content from values within the normal range to values more than ten times the upper limit of the normal range.

Differentiations based on immunological studies stem back to the work of Bayne-Jones and Wilson (1922) and Robinson (1927). The earliest workers in the field concluded that the myeloma proteins were not a single entity but a group differing one from another. This has been largely confirmed by more recent work by Wuhrmann, Wunderley and Hassig (1950), who stressed the individual specificity of the myeloma globulins, more particularly those having mobilities of the same order as beta globulins. Later work by Slater, Ward and Kunkel (1955),

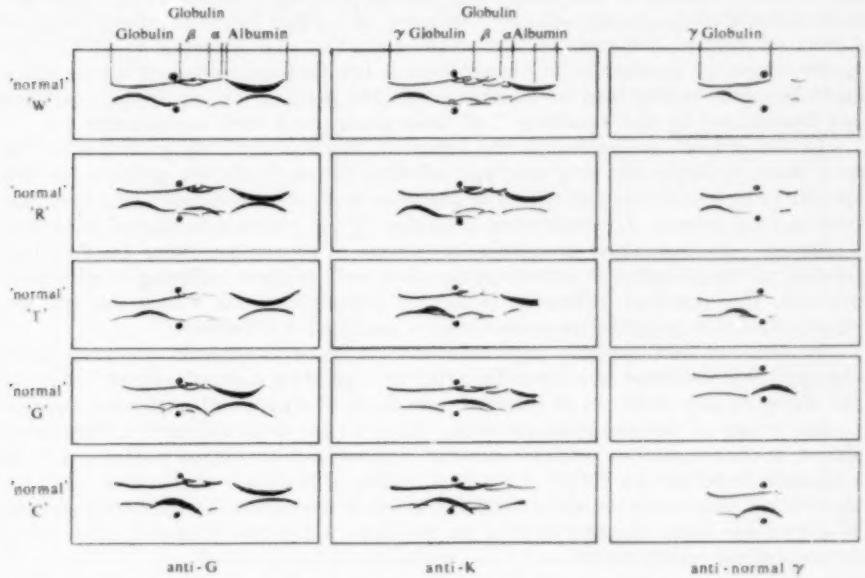

FIG. 4.—Tracing of immunoelectrophoretic analysis of sera "W," " R," " T," " G," and " C." The black dots represent the wells in which 0·001 ml. serum dilution, equivalent to 5 g./100 ml. of protein were placed. The electrophoretic analyses were carried out at 15 volts across each slide for four hours in glycine buffer, 0·05 ionic strength, with 2 per cent agar as support medium. At the completion of the electrophoretic analyses, the specific antisera were allowed to diffuse out through the agar from saturated strips of filter paper, placed centrally on the slide in its long axis.

employing precipitin techniques and the diffusion technique of Ouchterlony, showed, among other features, that "the gamma type of myeloma proteins were all related to normal gamma globulins or a fraction thereof . . . " while there was " . . . a definite but distant relationship between the beta myeloma proteins and normal gamma globulin."

In so far as they are comparable, the immunoelectrophoretic studies presented in this paper confirm this view, suggesting that antigenic sites of the myeloma globuli have much in common with normal gamma globulin.

The recent work on rabbit gamma globulin by Porter (1959) seems to show that antigenic activity is confined to one fragment of the whole molecule comprising approximately 45 per cent of the whole unit. If the antigenic sites of human gamma

globulin are concentrated in the same way, it is not difficult to collate the physico-chemical differences with the immunological similarities in this complex group of proteins.

The production of a " biologically " satisfactory protein takes place in three phases, the assemblage of aminoacids and their linkage through the usual condensation of the terminal carboxyl of the one with the alpha amino with adjacent member in the series, the total or partial coiling of the chain so formed into a helix and the final folding of this helix ; the second and third phases being associated with cross-bonding giving —S—S— bridging, and other forms of cross-linkage of a less well-defined and perhaps less stable character. The effect of this folding is to determine the number of reactive groups on the outward surface of the molecule and their relationship one to another. Specific antigen and antibody reactions are determined by the " pattern " of these groups and their accessibility.

In the present examinations the antibody reaction with the globulins in the individual myeloma sera was constant, whether the antibody was produced to the specific globulins of that patient, or to globulins from other patients, or to globulins from normal people. It would seem then that, if this reaction is related to surface " pattern " on individual molecules or groups of molecules, these patterns are present in the globulins in normal patients as well as those suffering from myelomatosis, the essential difference being the frequency with which one pattern recurs, that is a quantitative rather than a qualitative difference.

In the hundred cases of myelomatosis we have examined, we have never obtained any evidence of a hereditary defect suggesting a genetic origin. Nor, so far, have we any evidence of internal variations of aminoacid sequences peculiar to one or any of the myeloma proteins. Many of the demonstrated differences in shape, in charge, and in predominance of a given immunological pattern, may be explicable in terms of a defect of the final folding of the chain rather than in variations in the aminoacid sequence similar to those demonstrated in the haemoglobins. It is possible these changes depend on the ratio within the molecule of a helical to non-helical configuration and this possibility is being tested.

SUMMARY

(1) Abnormal globulins of varying mobilities have been examined from five patients afflicted by myelomatosis.

(2) The ultracentrifugal and immunochemical analyses suggest that these globulins show a progressive deviation from the normal rather than falling into well defined groups. The cause of this deviation is discussed.

Our thanks are due to the British Empire Cancer Campaign who defrayed the cost of this work.

REFERENCES

Bayne-Jones, S. and Wilson, D. W.—(1922) *Johns Hopk. Hosp. Bull.*, **33**, 119
Franglen, G. T., Martin, N. H. and Treherne, J. D.—(1955) *J. clin. Path.*, **8**, 144.
Koenig, V. L. and Pederson, K. O.—(1950) *Arch. Biochem.*, **25**, 97.
McConnell, R. J. and Martin, N. H.—(1958) *Brit. J. Cancer*, **12**, 264.
Mcfarlane, A. A.—(1935) *Biochem. J.*, **29**, 1175.
Müller-Eberhard, F. and Kunkel, H. G.—(1956) *J. exp. Med.*, **104**, 253.

NEURATH, H. AND BAILEY, K.—(1953) 'The Proteins,' Vol. 1, Section B. New York (Academic Press), p. 659.
PORTER, R. R.—(1959) *Biochem. J.*, **73**, 119.
PROOM, H.—(1943) *J. Path. Bact.*, **55**, 419.
PUTNAM, F. W.—(1956) *J. cell. comp. Physiol.*, **47**, 17, Suppl. i.
Idem AND UDIN, B.—(1953) *J. biol. Chem.*, **202**, 747.
ROBINSON, A.—(1927) *Brit. J. exp. Path.*, **8**, 454.
SCHEIDIGGER, J. J.—(1955) *Int. Arch. Allergy, N.Y.*, **7**, 103.
SLATER, R. J., WARD, S. M. AND KUNKEL, H. G.—(1955) *J. exp. Med.*, **101**, 85.
TAYLOR, J. F.—(1952) *Arch. Biochem. Biophys.*, **36**, 357.
WALLENIUS, G., TRAUTMANN, R., KUNKEL, H. G. AND FRANKLIN, E. C.—(1957) *J. biol. Chem.*, **225**, 253.
WEITZ, B.—(1952) *J. Hyg.*, **50**, 275.
WUHRMANN, F. H., WUNDERLY, C. L. AND HASSIG, A.—(1950) *Brit. J. exp. Path.*, **31**, 507.

CELL DIVISION IN AN ASCITES TUMOUR *IN VITRO*

With Especial Reference to Abnormalities of Cytoplasmic Cleavage

D. C. ROBERTS and D. J. TREVAN

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Received for publication October 1, 1960

The concept of stem lines within populations of tumour cells is now well established, having first been put forward by Makino and Kano (1953) and by Hauschka (1953) on the basis of experiments with ascites tumours of rats and of mice respectively. According to this concept there are within certain tumour-cell populations one or more lines of stem cells which are capable of regular mitosis and which are the primary contributors to the growth of the tumour, while outside the stem lines there is a variable proportion of genetically unbalanced cells, the whole population being in a state of dynamic equilibrium which may be altered by appropriate stimuli (Hauschka *et al.*, 1956).

The purpose of this paper is to record the behaviour of cells from sublines of the derived ascites form of a naturally occurring murine epithelioma, at and about the time of their division, and to draw attention to the lability of the minority of cells in the population which divide in an abnormal manner under the conditions of our experiments.

MATERIALS AND METHODS

The tumour cell populations used in these studies are from two derived ascites sublines of the tumour Epithelioma 255. Epithelioma 255 was found on the skin of the flank of a 2¼-year-old stock male C/57 mouse in 1955; it proved to be a well differentiated squamous cell carcinoma, and since that time has been maintained by one of us (DT) in mice of the strain of origin.

The standard culture medium consists of 16 per cent calf serum in Hanks' solution containing 0·07 per cent each of sodium bicarbonate and glucose and 0·35 per cent lactalbumin hydrolysate ("Bacto-Lactaltone", Difco) expressed as percentages of the total volume of medium. No antibiotics are used. This medium has a pH of 7·1 when in equilibrium with 5 per cent carbon dioxide.

Before preparing a population of tumour cells for examination, all apparatus, instruments and solutions required are placed in a hot room at 36·5° C. and allowed to come to temperature. A mouse bearing an ascites subline of Epithelioma 255 is killed, pinned to a cork board and immediately transferred to the hot room in which all subsequent manipulations are done. The abdominal skin is reflected and 0·1 ml. of ascites fluid is drawn into a syringe already containing 0·9 ml. of culture medium. This is mixed and one drop is expelled into 1 ml. of culture medium. The resulting dilution (about 1 in 250) is mixed and drawn up into a second syringe.

A sample of the resulting suspension of tumour cells is introduced into a culture chamber (Roberts and Trevan, 1960) which is placed on the stage either of a cinemicroscope in the same hot room (Trevan and Roberts, 1960; Pybus, 1960) or of a transportable cinemicroscope (Pybus, Roberts and Trevan, 1960) also at 36·5° C. which is kept in a neighbouring laboratory. The drops in the chambers are maintained throughout the experiment in equilibrium with a water-saturated atmosphere of 5 per cent carbon dioxide in air, and the cultures are fed at appropriate intervals (usually every 1–3 days). Phase contrast optics are used on the cinemicroscopes and time-lapse cinephotomicrographic records are taken continuously throughout the course of the experiment on 16 mm. slow pan negative film at the rate of one frame every 24 or 48 seconds.

The cine records are analysed in the first instance as negatives (we develop our own film but normally have negative film printed only when we require to assemble, in one film, sequences from the records of a number of experiments), a standard 16 mm. projector with a 1 inch projector lens and a throw of 12 feet being used. We find that the angle subtended to the eye is too large for the accurate examination of the whole field of view on the screen at one time; the screen therefore is divided into 35 squares of equal size and a film is shown either to a single observer for the requisite number of times, or once to several observers who each critically examine a part of the field.

A recent acquisition is a Muray 16 mm. film editor which, used in conjunction with a Premier footage and frame counter, is proving invaluable for the analysis of complex sequences and indispensable when events have to be timed.

RESULTS

When first explanted and viewed on the phase microscope the cells appear as spherical bodies with no discernible internal structure surrounded by a thin rind of cytoplasm which at times shows undulating movements. Later in the experiment most of the cells flatten on the glass, and much internal structure can then be seen; nuclei, perinuclear granules and mitochondria are visible and the cytoplasm is bordered by an undulating membrane. In some circumstances the cells orientate themselves one to another and make intercellular contacts of a kind never seen between cells in the rounded form.

When a cell divides in a " normal " manner it is as follows. The cell suddenly releases all firm contacts with the glass substrate and with any surrounding cells and the cytoplasm quickly shrinks until the cell appears as a spherical body in which little or no internal structure can be seen. After a varying period of complete immobility the spherical body elongates slightly and an indentation appears on either side which deepens until two apposed daughters are formed. The cells now quite suddenly flatten out, the internal structure can again be seen, an undulating membrane reappears at the edge of the cytoplasm and once more the cells can move by their own efforts or make cohesions with their fellows according to the conditions of the experiment. In cells dividing parallel to the plane of the glass, it is common for the two daughter cells to spread out simultaneously, although sometimes one expands before the other.

An expanded cell is usually attached much more firmly to the glass substrate than a rounded cell. In the event of a cell dividing at right angles to the plane of glass, the daughter in contact with the glass completes a " normal " division;

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An expanded cell is usually attached much more firmly to the glass substrate than a rounded cell. In the event of a cell dividing at right angles to the plane of glass, the daughter in contact with the glass completes a " normal " division;

that away from the glass, however, remains spherical with no discernible cytoplasmic movement, yet shifting as a whole in a quick and aimless manner as if wafted about by the movements of the underlying expanded cells. Immediately the spherical cell touches the glass, it flattens out.

Sometimes when a member of a sheet of cells rounds up to divide it is extruded onto the upper surface of the sheet, apparently by the pressure of the cytoplasmic borders of the surrounding, firmly attached flattened cells. Again the rounded daughter cells make no attempt to spread until they make contact with the glass substrate. Thus we can determine a period during which the cytoplasmic properties of an ascites cell differ from those of its interphase neighbours in an identical environment. This we term the functional mitotic interval : it is the period between the rounding up of a cell before division and the spreading out of a daughter cell after cleavage. The interval may conveniently be divided into two parts ; the period from the rounding up of the cell to the beginning of cytoplasmic constriction and from that constriction to the spreading of a daughter cell.

Fig. 1 shows a distribution graph of the functional mitotic intervals of the 243 cells in division that we have analysed ; we have excluded from this figure cells that lie on top of the sheet as the time at which these expand appears to be dependent on chance contact with unoccupied substrate rather than a function of the physiological state of the cells. It will be seen that the functional mitotic intervals of the cells that undergo " normal " division show a wide range of duration to produce a skew curve with a long tail in the high time range and a mode at 51–60 minutes. Although the numbers are small, it appears that the functional mitotic intervals of the cells that have been seen to divide abnormally probably follow the same pattern as those of the cells that divide " normally ".

ABNORMALITIES OF CYTOPLASMIC CLEAVAGE

Fig. 2 indicates diagrammatically the abnormalities of division which we have seen, and roman numerals in the text refer to this figure.

The most simple abnormality of cleavage recognised was the persistence of a thread of cytoplasm joining two daughter cells when they spread out after the division of a uninucleate cell ; this thread parted to form two free daughter cells (II). In this series such cytoplasmic threads were seen on 8 occasions, and the times from the spreading out of a daughter cell to the breaking of the cytoplasmic thread varied between 18 and 43 minutes except in one cell when the thread remained for 138 minutes.

Sometimes a cytoplasmic thread failed to break ; and instead thickened until the two daughter cells came together to form a binucleate cell (III). This we have seen on 7 occasions, the time between the spreading of a daughter and the formation of a recognisable binucleate cell varying between 42 and 324 minutes. We were unable to foresee from the appearance of a thread whether it would break with the formation of two separate daughters or persist with the formation of a single binucleate cell. A similiar process was seen in 7 cells in which there was either an abortive attempt or apparently no attempt at cytoplasmic cleavage, and the cell, which was uninucleate when it rounded up, was seen to be binucleate when it re-expanded (IV).

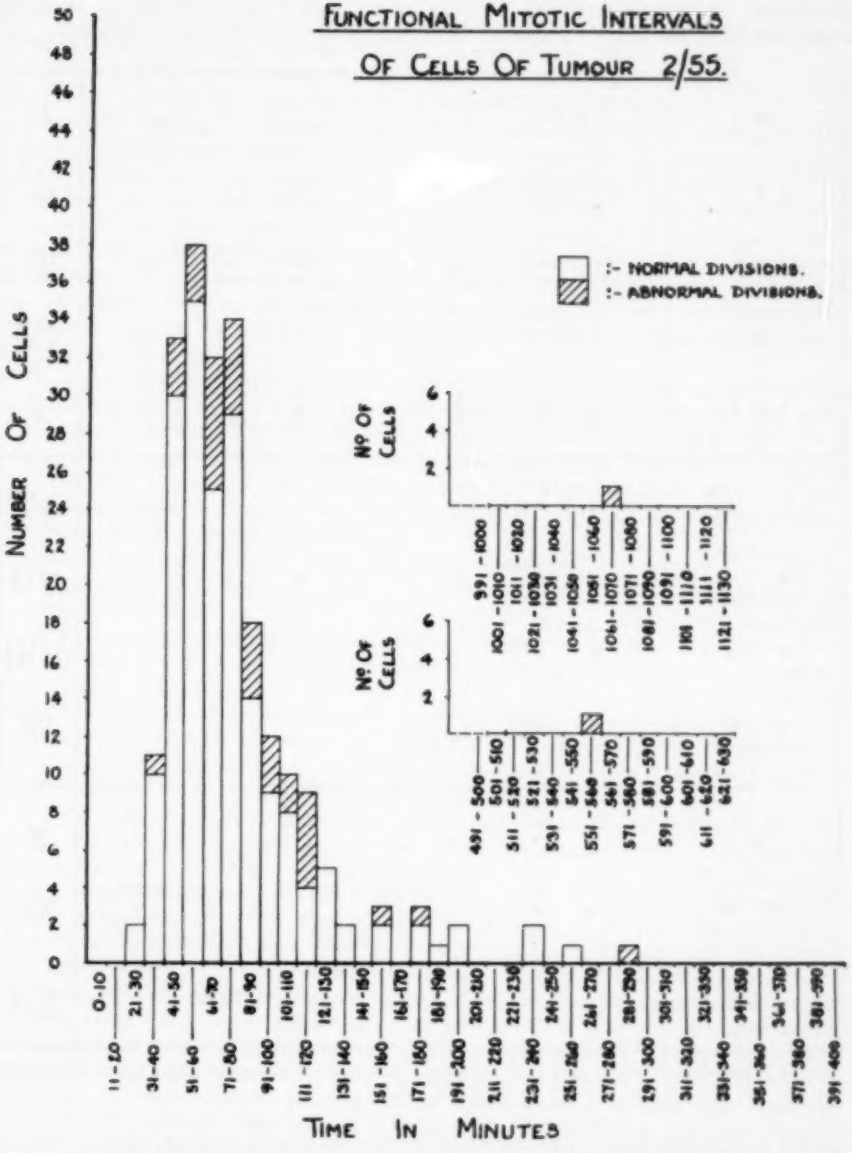

FIG. 1.—Distribution graph of the functional mitotic intervals of ascites cells of Epithelioma 255.

Another mode of formation of a binucleate from a uninucleate cell was seen once only. A uninucleate cell rounded up to divide and cytoplasmic cleavage began 23 minutes later. Nineteen minutes after this the first daughter cell

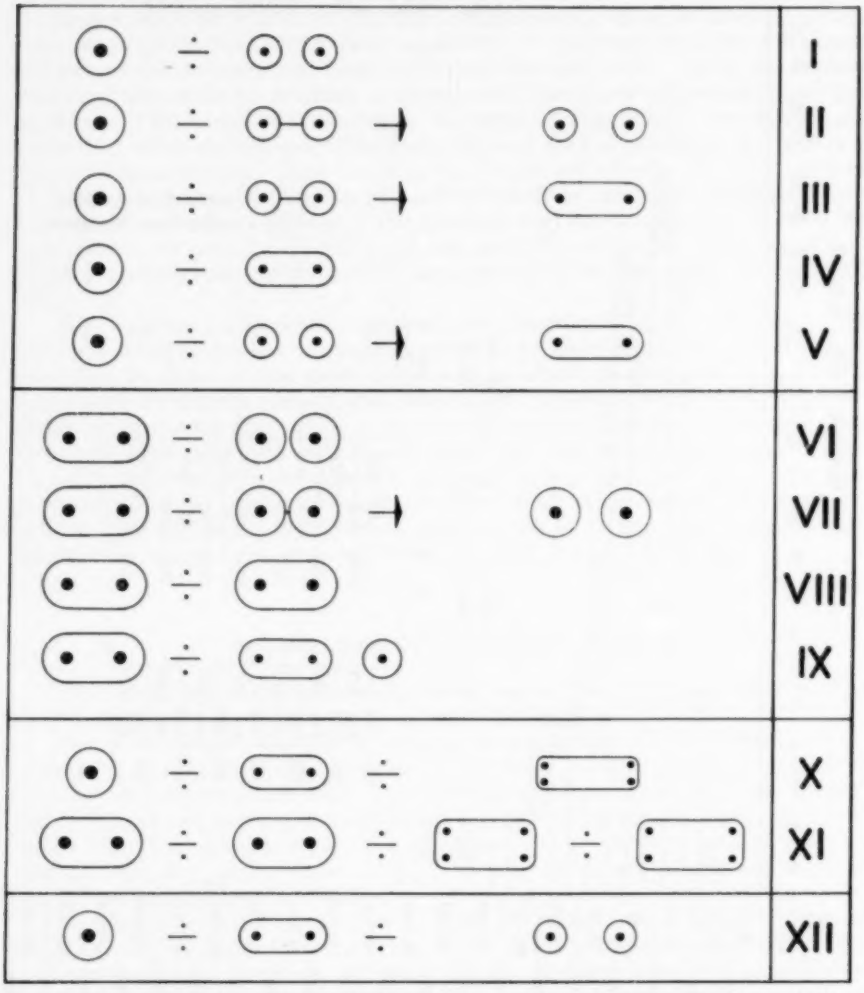

FIG. 2.—Diagram of abnormalities of cytoplasmic cleavage seen in ascites cells of Epithelioma 255.

spread out on the glass to be followed by the second 14 minutes later. The two daughters appeared to be entirely separate from one another and despite repeated examination of the cine record no connecting thread has been seen. After a further 554 minutes however, the two cells came into close apposition and their

cytoplasms fused to form a single binucleate cell (V). A binucleate cell is not necessarily committed to the binucleate state in future generations; indeed we have not seen a binucleate cell that has produced two binucleate daughters at division. On 8 occasions we have observed binucleate cells round up to divide, undergo successful cytoplasmic cleavage and give rise to two uninucleate daughter cells (VI). On only three occasions have we seen a binucleate cell that attempted division and then again formed a single binucleate cell. In the first case cytoplasmic cleavage was nearly completed. The binucleate cell rounded up and cleavage began after 30 minutes; the first daughter cell flattened out after a further 56 minutes, and the second 58 minutes after that. The cells were seen to be joined by a cytoplasmic thread which disappeared after 83 minutes and a single binucleate cell was again formed (VII). In the second case a binucleate cell rounded up, 62 minutes later an abortive attempt at cleavage was seen and it was not until a further 490 minutes had elapsed that the cell spread out again, still containing two nuclei (VIII). The third cell was seen to divide several times, and will be considered later.

Only once have we seen the formation of one binucleate and one uninucleate cell from the division of a single binucleate cell. This cell rounded up and after 75 minutes underwent a 3-way division. After a further 45 minutes, two of the daughter cells expanded to be followed by the third after a further 69 minutes. This third cell was seen to be joined to one of the other daughter cells by a thin cytoplasmic thread which persisted for 45 minutes; it was then absorbed and a binucleate daughter was formed (IX).

Successive divisions each with failure of cytoplasmic cleavage give rise to very big cells with large and complex nuclear systems, although this may be followed by the death of the cell. Thus we have seen a uninucleate cell which rounded up and cytoplasmic cleavage began 14 minutes later; cleavage was not complete and 140 minutes afterwards the cell spread out again in a binucleate state. After a further 1062 minutes the cell rounded up again and an early cytoplasmic furrow was seen after 45 minutes. Sixty-six minutes later the cell attempted to divide, failed, remained as a single cell and after another 285 minutes expanded to reveal 4 nuclei. It appeared to be quite healthy, but after a further 231 minutes the cytoplasm suddenly coagulated and it died (X).

Another cell contained two nuclei when first seen. It rounded up for the first time and a cytoplasmic furrow was visible 51 minutes later. Cytoplasmic cleavage did not take place and after a further 38 minutes it expanded again, still in a binucleate state. It remained spread for 928 minutes and then rounded up for a second time; after 41 minutes a furrow could be seen in the cytoplasm, but there was no cytoplasmic cleavage and 60 minutes later the cell again flattened out on the glass, this time with 4 nuclei. The cell remained expanded for 1141 minutes after which it rounded up for a third time; a cytoplasmic furrow was seen after 74 minutes followed by an attempt at a 4-way division which failed; after a further 46 minutes the cell spread out once more, still with 4 nuclei (XI).

Failure of cytoplasmic cleavage in a uninucleate cell does not preclude successfull cytoplasmic cleavage in a later generation. Twice we have seen a uninucleate cell which rounded up to divide, failed to cleave and produced a binucleate cell which subsequently divided successfully into two uninucleate daughters (XII).

DISCUSSION

The cell preparations used in this study were prepared in such a way as to give the best possible visualisation of the cytoplasm. The refractive index of the culture medium was too low to permit analysis of the behaviour of the chromosomes during cell division, and we do not propose to discuss here the effects on ploidy that may result from these aberrant cell divisions. The examination of cells in a high protein medium as described by Barer and Joseph (1955) might well allow the nuclear behaviour of similar cells to be studied and correlations determined. Comparisons could then be made with the modes of formation of binucleate and polyploid cells in non-neoplastic tissues as described in the regenerating liver of the rat by Beams and King (1942) and in cultures of mouse fibroblasts by Fell and Hughes (1949).

The picture that emerges from this *in vitro* study is that of a population of cells in which the great majority of cells that divide do so in an apparently normal manner while a minority show a wide range of abnormalities of cytoplasmic cleavage. The cells resulting from these abnormal divisions are usually viable and have frequently been seen to undergo further division which may appear to

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EXPLANATION OF PLATES

FIG. 3–5.—Formation of a binucleate cell from a uninucleate cell.
FIG. 3.—A uninucleate cell (A) before division. × 280.
FIG. 4.—Daughter cells joined by a cytoplasmic thread. × 280.
FIG. 5.—The thread has been absorbed with the formation of a single binucleate cell. × 280.

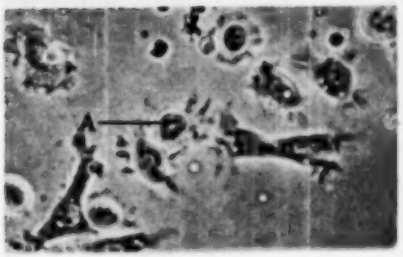
FIG. 6–7.—Formation of a binucleate cell from a uninucleate cell.
FIG. 6.—A uninucleate cell (B) before division. × 280.
FIG. 7.—Resulting binucleate cell from (B). × 280.

FIG. 8–11.—Formation of a binucleate cell from a uninucleate cell.
FIG. 8.—Uninucleate cell (C) before division. × 330.
FIG. 9.—Two apparently separate daughters (D) and (E) from the division of (C). × 330.
FIG. 10.—Fusion taking place between (D) and (E). × 330.
FIG. 11.—A binucleate cell (G) resulting from the fusion of (D) and (E). × 330.

FIG. 12–15.—A uninucleate cell which divides to form a single binucleate cell. This cell then divides to form two uninucleate daughters.
FIG. 12.—Uninucleate cell (H) before division. × 330.
FIG. 13.—Binucleate cell (I) resulting from division of (H). × 330.
FIG. 14.—Binucleate cell (I) just before its division. × 330.
FIG. 15.—Two uninucleate cells (J) and (K) resulting from the division of (I). × 330.

FIG. 16–19.—Formation of one uninucleate and one binucleate daughter from the division of a single binucleate cell.
FIG. 16.—Binucleate cell (L) before division. × 280.
FIG. 17.—Three-way division of cell (L). × 280.
FIG. 18.—Daughters from division of cell (L). Two daughters are expanded while one is still rounded up. × 280.
FIG. 19.—Binucleate cell (M) and uninucleate cell (N) formed from the division of cell (L). × 280.

FIG. 20–23.—Formation of cell with complex nuclear structure from a binucleate cell.
FIG. 20.—Binucleate cell before division. × 330.
FIG. 21.—The cell has attempted division, there has been failure of cytoplasmic cleavage, and a binucleate cell is again formed. × 330.
FIG. 22.—Attempted division has again taken place; cleavage has again failed and a single 4-nucleate cell has been formed. Only three of the nuclei are visible in this figure. × 330.
FIG. 23.—The 4-nucleate cell has attempted division, cleavage has again failed, with the formation again of a cell with 4 nuclei. × 330.



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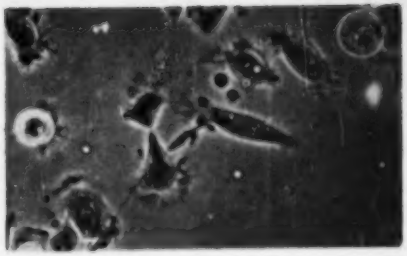

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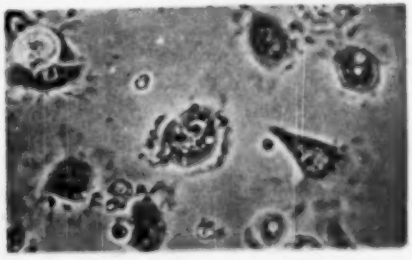

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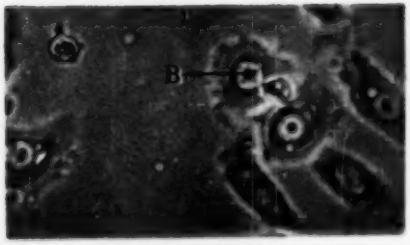

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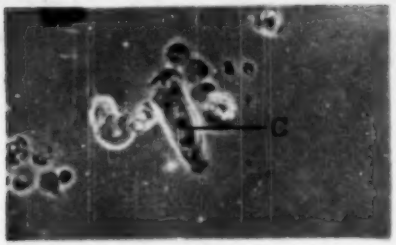

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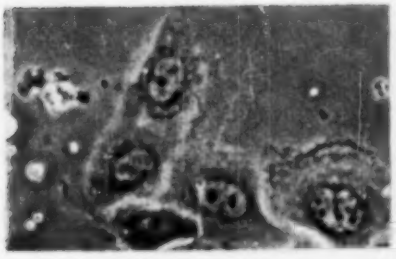

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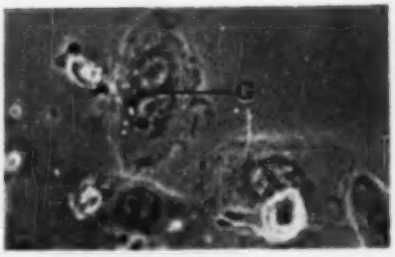

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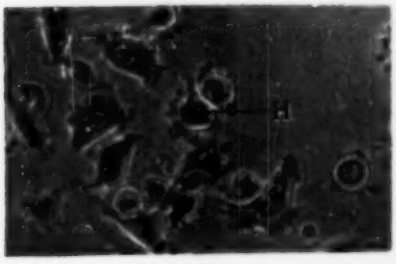

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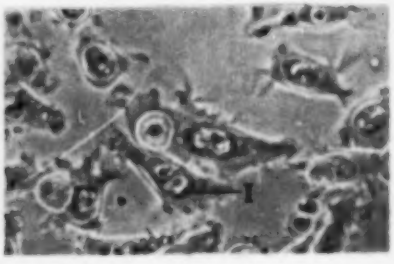

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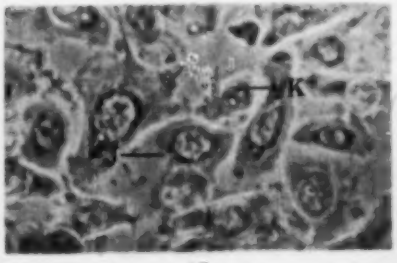

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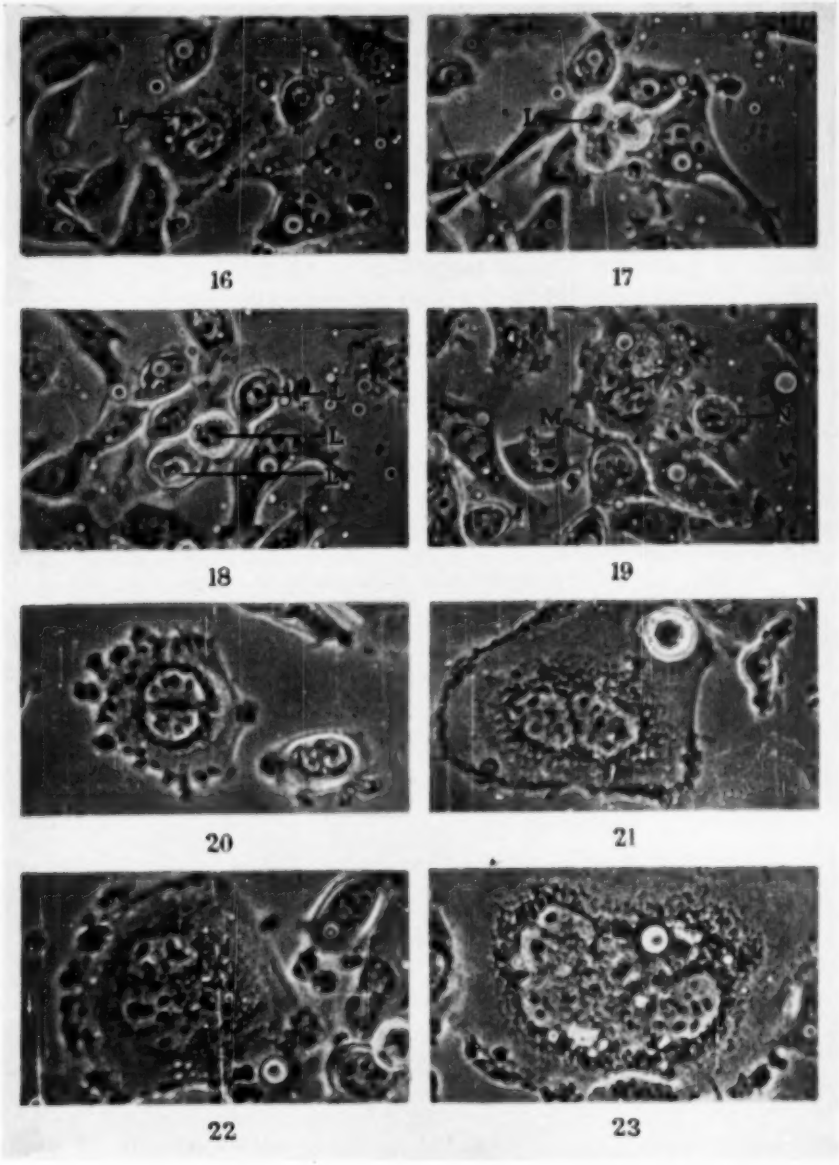

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be normal or may again show abnormalities of cleavage ; a minority population of variant cells is thus produced the members of which may themselves differ either qualitatively or quantitatively from generation to generation.

Klein and Klein (1956) and Hauschka (1953) have shown that progression of a tumour may be brought about by a selective process acting within a tumour cell population whereby a variant cell line is raised numerically above a certain critical proportion of the population. This variant line can then displace competitive cell lines and become manifest by changing the characteristics of the tumour.

We do not know what environmental or other factors determine the content of the minority cell population that we have described, nor whether the changes that we have observed *in vitro* reflect similiar behaviour *in vivo* or represent potentialities of the cells that remain unfulfilled in the absence of special factors in the animal host. It is possible, however, that this changing minority population may represent a mechanism whereby variant cells may arise, which, if environmental factors are such as to give them a selective advantage over the rest, may be the progenitors of a new stem cell line.

SUMMARY

This paper records the behaviour *in vitro* of cells of an ascites subline of a naturally occurring murine epithelioma at and about the time of their division. Although the majority of cells divided in what appeared to be a normal manner, abnormalities of cleavage in a minority resulted in a minority population of variant cells the content of which might alter from generation to generation. The possible significance of these findings to the stem cell concept of tumour growth is discussed.

We wish to record our indebtedness to Dr. H. B. Fell, F.R.S., both for her encouragement of our work and for her help in the presentation of the results.

REFERENCES

Barer, R. and Joseph, S.—(1955) *Quart. J. micr. Sci.*, **96**, 1.
Beams, H. W. and King, R. L.—(1942) *Anat. Rec.* **83**, 281.
Fell, H. B. and Hughes, A. F.—(1949) *Quart. J. micr. Sci.*, **90**, 355.
Hauschka, T. S.—(1953) *J. nat. Cancer Inst.*, **14**, 723.
Idem., Kvedar, B. T., Grinnel, S. J. and Amos, D. B.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 683.
Klein, G. and Klein, E.—(1956) *Ibid.*, **63**, 640.
Makino, S. and Kano, K.—(1953) *J. nat. Cancer Inst.*, **13**, 1213.
Pybus, W.—(1960) *J. R. micr. Soc.*, **79**, 369.
Idem, Roberts, D. C. and Trevan, D. J.—(1961) *Ibid.* (in press).
Roberts, D. C. and Trevan, D. J.—(1960) *Ibid.*, **79**, 361.
Trevan, D. J. and Roberts, D. C.—(1960) *Ibid.*, **79**, 367.

SHEET FORMATION BY CELLS OF AN ASCITES TUMOUR *IN VITRO*

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IN the course of a study of the behaviour *in vitro* of cells from an ascites epithelioma of the mouse, it was noticed that the cells could aggregate into sheets of varying stability. This paper describes the phenomenon and some of the factors by which it is influenced.

MATERIALS AND METHODS

The cells to be described are from one of several ascites lines derived by deliberate selection from a murine epithelioma. This tumour arose on the back of an untreated male C/57 black mouse 2¼ years old. It has been maintained since 1955 by serial grafting in mice of that strain to which it is specific. It is designated "Epithelioma 255"; the details of its changing histology following different selective grafting procedures will be described elsewhere by one of us (DT). The ascites line concerned in this account is designated "B1". All ascites lines are maintained by serial intraperitoneal passage of peritoneal exudate diluted in phosphate saline to a degree depending on a rough visual estimate of cellularity, usually between 1 in 3 and 1 in 20.

Mice bearing the ascites line B1 show evidence of peritoneal exudate at from 10 to 14 days after inoculation. They have been killed to yield cells for study at times after inoculation ranging from 10 to 38 days, but usually at about 18 days. Typically at this time there is one to 3 ml. of exudate containing about 10^5 cells per cu. mm. and an amount of blood estimated visually at between 5 and 25 per cent of the volume. Solid tumour is present as a craggy mass about 20 × 5 mm. in the lesser omentum and around the pancreas, as multiple nodules in the mesentery and as a thin sheet on the parietal peritoneum. There are multiple minute secondary deposits and haemorrhages in the lungs. Mice killed earlier show less ascites often with less blood and less solid growth; at 10 or 14 days there are usually haemorrhages in the lungs but only microscopic metastases. Mice surviving beyond about 18 days often accumulate up to 7 or 8 ml. of ascites and much more solid growth, while the lungs are grey and rigidly consolidated with secondary tumour. Lung haemorrhages tend to be less severe in long survivors.

Cells for observation are taken from a mouse killed with coal gas a few minutes previously and kept warm, by aspiration into a pre-warmed siliconed syringe of medium. A secondary dilution to a final degree of about 1 in 250 is made in the same medium, which consists of Hanks' saline with 16 per cent of calf serum,

0·35 per cent of lactalbumin hydrolysate (Bacto-lactaltone, Difco), and 0·07 per cent each of sodium bicarbonate and glucose, all expressed as percentages of the whole medium. The pH of the medium is 7·1 when equilibrated with 5 per cent carbon dioxide in air at 37° C. The cell suspension is then injected into a chamber (Roberts and Trevan, 1960*a*) through which 5 per cent carbon dioxide in air passes, and the cells resting on the lower glass slip are examined with an inverted phase-contrast microscope at 37° C. and photographed by time-lapse cinematography. Dilution of the fluid is adjusted to produce a final concentration of cells of between 500 and 1000 per cu. mm., which in this chamber, in a drop 1·5 mm. deep, gives about 5 to 10 cells in the field of a ×20 objective at the start of an experiment.

RESULTS

In such cultures no constant differences in behaviour have been found between cells of the B1 subline taken from mice at different times after inoculation within the range of 10 to 38 days. The cells are at first spherical, floating singly or in small clumps or strings of from 2 to a dozen cells. They attach themselves to the glass, showing fine spiky projections of cytoplasm, often within half-an-hour of removal from the mouse. They then begin to spread out, but not simultaneously ; after 12 to 24 hours in culture most of them have assumed a flattened form and are moving about actively on the glass surface, from time to time becoming spherical again and dividing. The behaviour of these cells during division has been described by Roberts and Trevan (1960*b*). The flattened cells vary in shape. Some are discs, entirely surrounded by an actively amoeboid ruffled border. Others are unipolar, bipolar or multipolar, with areas of active ruffled border separated by smooth more refractile quiescent regions. The ruffled areas form pseudopodia and a cell tends to move in the direction of its most active pseudopodium.

When the pseudopodia of two cells meet, they do not usually both remain apposed and active. In many instances the ruffled border of one of the cells becomes inactive, smooth and refractile along the line of apposition. It then withdraws followed by the active border of the other cell, and may even become deeply concave with the ruffled border of the other cell within the concavity. In other cases first the ruffled border of one cell and then that of the other will alternately become inactive along the line of apposition, and this alternation of inactivity may be repeated several times. In the event of two ruffled borders coming into apposition and both remaining active, one of the cells moves laterally until it reaches an inactive part of the border of the other cell.

Cells in the spherical form do not cohere at any time in the life of the culture, except that, as already mentioned, the free-floating cells fresh from the peritoneal cavity are found in clumps. When these first attach themselves to the glass they separate as they spread out, and migrate apart ; evidently they adhere more strongly to the glass than to each other at this stage. They form loose aggregates, in which they behave as described in the previous paragraph, without cohering and without migrating over one another, so that a monolayer is maintained. This we have called a "sliding sheet" : at a single glance, or in a fixed and stained preparation, it might seem that the cells are cohering, but cinematography shows that this is not so.

If, however, the culture medium is not renewed, the cells in such aggregates form increasingly lasting attachments to each other during the next 12 to 30 hours, so that a culture between 40 and 60 hours old in which there has been no change of medium contains many areas in which cells that are still flattened on the glass now cohere, still as a monolayer, resembling normal epithelium. Here and there among the cells can sometimes be seen fine intercellular strands like those between normal prickle cells. Only the cells at the edge of the pavement still show active movements of their free borders, and there is a general diminution of motility, in spite of which a small sheet may move across the glass as a whole in a manner that demonstrates the firmness of the attachments between its members. A sheet increases in extent by the attachment of more cells migrating to it on the glass, and by cell division, which continues for at least 15 hours after the formation of such fixed sheets. If a change of medium is still withheld, the activity at the edges of the sheet gradually lessens until none can be seen. Ultimately the cells shrink, fall apart and die. A minority of the cells in the population never adhere to a fixed sheet when they meet it ; many of these are the giant, often multinuclear, cells produced by aberrations of division (Roberts and Trevan, 1960*b*). We have a film sequence that shows a stable sheet being formed as a result of several successive divisions in an initially small group of cells in contact. The daughter cells from each division adhered firmly and promptly to their neighbours in the sheet ; nearby were several larger very actively amoeboid tumour cells that adhered neither to each other nor to the growing sheet.

If the culture fluid is removed and replaced with fresh medium after the formation of fixed cell-sheets and before the death of the culture there is an immediate renewal of amoeboid activity and the adhesions between cells in the sheet break. The cells form a sliding sheet or migrate apart ; if division has ceased it begins again. As time goes on, however, adhesions again form between cells and unite them in stable sheets. This happens more rapidly than it did in the fresh culture, i.e., in about 10 as against 30 hours ; the culture is now more crowded than it was when fresh, and the more crowded a fresh culture is the more quickly stable sheets are formed. A stable sheet re-formed after feeding the culture usually breaks up again at the next feed, but less completely, and it re-forms even more quickly than before. If the medium is renewed every day, stable sheet formation may be postponed at least until the point when the culture becomes so crowded that many cells die in spite of the frequent changes of medium. Cells in contact remain in sliding sheets throughout.

On three occasions stable sheets have been subjected to the replacement of the culture fluid with medium from which the glucose has been omitted. The sheets remained stable and adhesions between the cells did not break.

DISCUSSION

When the cells of this ascites tumour derived from a well differentiated murine epithelioma are maintained in culture on a glass surface in a good state of nutrition they flatten, adhere to the glass, and migrate upon it. When they meet they slide around but not over one another, remaining in a monolayer, but they do not cohere. When pseudopodia of two cells meet, one of the pseudopodia may inhibit the activity of the other, as though it were "dominant". Such

" dominance " is however often repeatedly reversed. This behaviour is not contact inhibition, described by Abercrombie and Heaysman (1954) as the normal behaviour of fibroblasts in culture, and as responsible for the maintenance of a monolayer : contact inhibition is defined as being associated with cell cohesion, and is believed to depend upon it. Abercrombie, Heaysman and Karthauser (1957) later reported that sarcoma cells failed either to show contact inhibition or to remain in a monolayer when confronted with other sarcoma cells or with fibroblasts. Thus the behaviour of Epithelioma 255 cells when they form non-coherent monolayers may be regarded as intermediate between these two extremes ; they inhibit one another's movements more than sarcoma cells do, but do not cohere. This is reminiscent of the observations of Weiss and Taylor, quoted by Weiss (1958) of aggregates of normal epithelial cells. Moscona (1957) had found that suspensions of dissociated embryonic epithelium taken at a stage when differentiation was established would reaggregate according to histological type, even if the cells were from different species. Weiss and Taylor made cinematographic records of the re-aggregation process in order to discover what part if any was played by specific adhesion between cells of like type. Weiss concluded that " Cells in homologous, as well as heterologous, groups keep shifting about one another in a manner which rules out stable mutual attachments as a primary event in aggregation. The only difference between the homologous and heterologous groups seems to be that homologous cells tend to retain mutual contact along a broad front whereas heterologous combinations tend to break up soon after contact ". He illustrates the break-up of a heterologous combination by a photograph of a pseudopodium of a liver cell within a smooth concavity on the surface of a lung cell ; this strongly resembles what may happen when two cells of Epithelioma 255 meet ; on the other hand, these epithelioma cells also form aggregates, which we have called " sliding sheets ", which resemble the aggregates of homologous epithelium seen by Weiss and Taylor, in that " they retain contact along a broad front " without cohering.

The " dominance " of one pseudopodium over another might be explained in the following way. A cell puts out a pseudopodium by virtue of its contact with, and adherence to, the substrate. The advancing edges of opposed pseudopodia are thin and sharp ; when they meet either edge may undercut the other and loosen it from the substrate so that it retracts elastically and progressively from the edge inwards. Further advance of the undercutting pseudopodium detaches more of the other from the substrate ; if this advance ceases the undercut cell margin has an opportunity to spread out again and may undercut the other cell in turn. It may be a matter of chance which pseudopodium first undercuts the other. Once a cell border is undercut it becomes blunter and is thus less likely to undercut the other cell border until it can spread out again ; this it cannot do until there is a clear space on the substrate to which it can adhere. The "dominance" we have seen applies at present only to pseudopodia on a glass substrate ; we have so far not observed the behaviour of these cells in contact on other substrates or floating free.

If the medium in a culture of Epithelioma 255 ascites cells at the " sliding sheet " stage is not replenished, the cells begin to form more and more lasting adhesions to one another without at first any decline in migratory activity, so that they may move over the substrate in small compact groups. This shows that the formation of sheets of coherent cells is not entirely attributable to the

gradual lessening of migratory activity that in the end accompanies relative starvation. This behaviour is analagous to contact inhibition. Abercrombie, Curtis and Karthauser (1958) have reported that some failure of contact inhibition may be produced in cultures of fibroblasts by a high concentration of embryo extract. Our observations of Epithelioma 255 also suggest a connection between nutrition and contact behaviour. There is no embryo extract in our medium, but the administration of medium containing little or no glucose to a stable sheet of cells of Epithelioma 255 does not cause it to break up ; this may indicate that the formation of intercellular adhesions is due to a reduction, by consumption, of the glucose available. Alternatively the cohesiveness may be induced by the consumption of some other nutrient or the production of some metabolite, and possibly the cells fail to part company when fed with medium lacking glucose because they have not enough energy to migrate apart ; the effect of glucose-free medium on cells in the early, actively migrating cohesive state has not yet been tried.

Dabrowska-Piaskowska (1959) applying Moscona's techniques to tumours found that cells of a murine mammary tumour reduced to a single-cell suspension with trypsin would re-aggregate into a structure resembling that of the tumour *in vivo*, when cultivated on glass or on a plasma clot. The sheets formed by Epithelioma 255 cells might be considered as analogous attempts at reconstruction, indeed, they show intercellular bridges which give them an at least superficial resemblance to prickle cells. The dependence upon nutrition *in vitro* of this apparent differentiation of the ascites form of Epithelioma 255 is of interest in that further study of the factors involved may throw some light on the mechanisms whereby the cells of a malignant tumour *in vivo* may vary in invasiveness or remain dormant for long periods.

SUMMARY

Cells of a murine ascites epithelioma when cultured on a glass surface in a medium consisting of calf serum, lactalbumin hydrolysate, salts, bicarbonate, and glucose adhere to the glass and move about on it. When two cells meet one may inhibit the pseudopodial activity of the other, but they do not cohere at this stage ; at a later stage, if the culture medium is not renewed, adhesions are formed between cells which eventually unite them into stable sheets resembling normal epithelium. Cells in these sheets may be connected by intercellular bridges like those between prickle cells. The sheets grow by accretion and by division of the cells in them. This behaviour is likened to the reconstruction of normal tissues and of tumours from dissociated cells in culture that has been observed by other workers.

The formation of stable sheets may be prevented or reversed by renewing the culture medium, and there is a little evidence that the medium must contain glucose in order to do this. The dependence of apparent differentiation upon environmental factors, possibly nutritional, justifies further study in an attempt to elucidate some of the mechanisms responsible for the variation in invasiveness of tumours *in vivo*.

We are very grateful to Dr. Honor Fell, F.R.S. for her interest in this work, and for her help with its presentation.

REFERENCES

ABERCROMBIE, M., CURTIS, A. S. AND KARTHAUSER, H. M.—(1958) *Rep. Brit. Emp. Cancer Campgn*, **36**, 507.
Idem AND HEAYSMAN, JOAN E. M.—(1954) *Exp. Cell. Res.*, **6**, 293.
Iidem AND KARTHAUSER, H. M.—(1957) *Ibid.*, **13**, 276.
DABROWSKA-PIASKOWSKA, KRYSTYNA.—(1959) *Ibid.*, **16**, 315.
MOSCONA, A.—(1957) *Proc. nat. Acad. Sci., Wash.* **43**, 184.
ROBERTS, D. C. AND TREVAN, D. J.—(1960*a*) *J. R. micr. Soc.*, **79**, 361.—(1960*b*) *Brit. J. Cancer*, **14**, 716.
WEISS, P.—(1958) "International Review of Cytology, vii, 391; Ed. Bourne and Danielli, New York (Academic Press).

FATE OF ^{35}S-CYSTINE IN MULTIPLE MYELOMA

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THE abnormal protein-bound polysaccharide associated with multiple myeloma (Sachs, Cady and Ross, 1954) and the handling of radiosulfate by patients with this disorder (Siegel, Sachs and Graig, 1959) have been reported previously. The present study describes the fate of ^{35}S labelled cystine in myeloma and other malignant neoplasms, and compares these results with those published earlier for radiosulfate.

MATERIALS AND METHODS

^{35}S as l-cystine (specific activity of about 30 mc/g.) in doses of approximately 0·5 mc was administered intravenously to 5 patients with multiple myeloma and to 5 patients with other neoplastic diseases (Table I). The assays of radioactivity were performed with a gas flow counter, since ^{35}S is a pure, weak beta emitter (0·168 mev maximum energy; 87 days half-life). Appropriate corrections for self-absorption and back-scattering were made for the specimen geometry employed as well as for physical decay. The detectors used were calibrated against ^{35}S standard solutions furnished by the National Bureau of Standards. Blood was collected by venipuncture at intervals of 5 minutes, 1 hour, 4 hours, 8 hours, 24 hours, 2 days, 3 days, 5 days, 7 days, and 14 days following administration of the tagged cystine. Blood specimens (about 15 ml.) were placed in test tubes containing two drops of versene to prevent coagulation. Urine was collected for the first 8 hours, the next 16 hours, and daily thereafter for two weeks after injection of ^{35}S cystine.

Calculations.—The ^{35}S concentration (per cent dose/liter) in whole blood and plasma (Fig. 1) and the fraction of the ^{35}S dose excreted (per cent dose excreted) in urine during 14 days following administration of the radioisotope were computed (Table I and Fig. 2). The apparent space of dilution was calculated from these blood and plasma data. This computation involved finding the hypothetical concentration at zero-time by means of graphical extrapolation in order to obtain the so-called zero-time radiosulfate space (Ryan *et al.*, 1956). The ratios of space to body weight and body surface were determined. Body surface of each subject was obtained from the patient's weight and height using the conventional Du Bois relationship.

RESULTS

^{35}S blood levels.—^{35}S in whole blood, as well as in plasma, falls rapidly for the first 8 hours and then declines slowly (Fig. 1). This pattern is found in both multiple myeloma patients and the other subjects. Since the levels and patterns are

almost identical in whole blood and in plasma, only whole blood data are given in Fig. 1. For whole blood, the initial maxima, obtained 5 minutes following the injection of tagged cystine, range from 9·39 per cent dose/liter to 16·9 per cent dose/liter for the myeloma patients and from 7·53 per cent dose/liter to 15·8 per cent dose/liter for the other group. At the same time, the corresponding plasma concentrations range from 8·96 per cent dose/liter to 17·1 per cent dose/liter for those with multiple myeloma and from 7·62 per cent dose/liter to 16·6 per cent dose/liter for the others. By 8 hours, these concentrations have dropped to about one-third of their respective 5 minute peaks. These levels decrease slowly thereafter, being about three-quarters of the 8 hour values at 5 days after the administration of the tagged cystine.

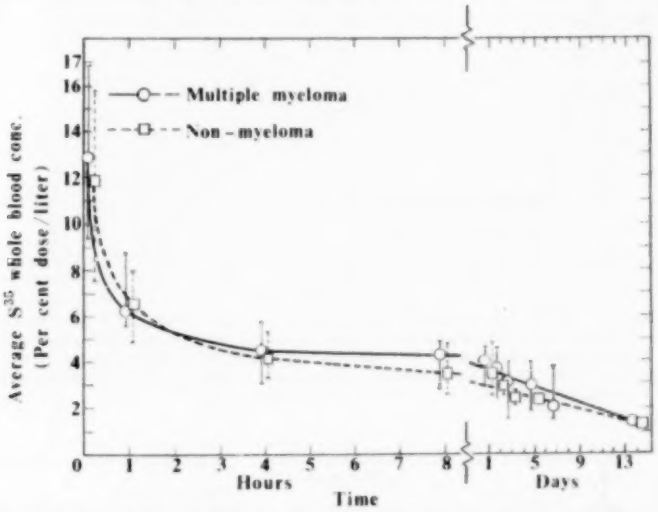

FIG. 1.—The variation of the mean ^{35}S whole blood concentration with time for five patients with multiple myeloma and five patients with other malignant neoplasms. The range (maximum and minimum values) is indicated by vertical lines extending from the individual points.

By graphing the logarithm of whole blood and plasma ^{35}S concentration against time, it was found that the curves thus obtained could be resolved into exponential components. The curves from three of five multiple myeloma patients have two decay rates. An initial rapid decrease, corresponding to a half-disappearance time (T_1) of about 0·45 hour characterizes the fall of ^{35}S during the first hour. The decline thereafter is quite slow, described by a half-disappearance time (T_3) of about 280 hours (Fig. 2). An intermediate half-time (T_2) of about 2·5 hours, however, was present for the other two myeloma patients during the 1 to 8 hour interval following the injection of the doses. For the other group of patients, three distinct rates governed the disappearance of the ^{35}S (Fig. 3). An initial half-time (T_1) of about 0·26 hour for the first hour is followed for the next 8 hours by an intermediate component (T_2) of about 1·4 hours. For the ensuing period, the gradual fall is described by a half-time (T_3) of about 160 hours. Mean values of

these half-disappearance times for both groups of patients, as derived from their respective whole blood and plasma curves, based on the data collected over a two week period, are listed in Table II. The figures reproduced here (Fig. 1 and 2) cover only the initial 24 hours following the administration of the labeled amino acid to illustrate more clearly the early phases.

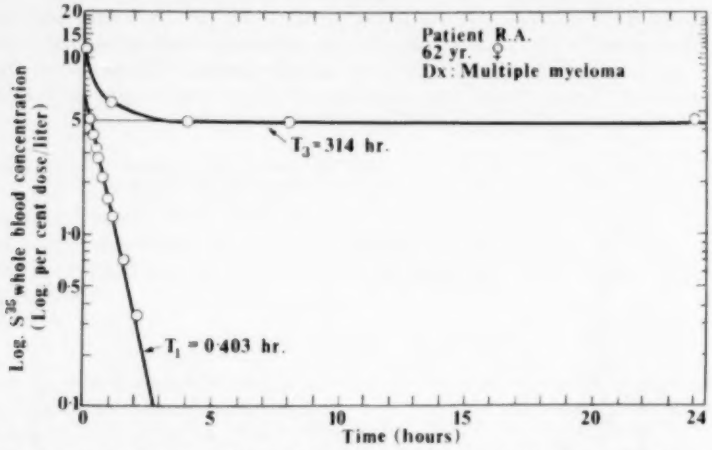

FIG. 2.—Resolution of the whole blood ^{35}S concentration curve obtained from a patient with multiple myeloma into two exponential components. T_1 and T_3 are the respective initial and late half-disappearance times. The various half-disappearance times given in the figures and Table II are actually based on graphs covering the two week period of observation; for illustrative purposes, only the early values are presented here.

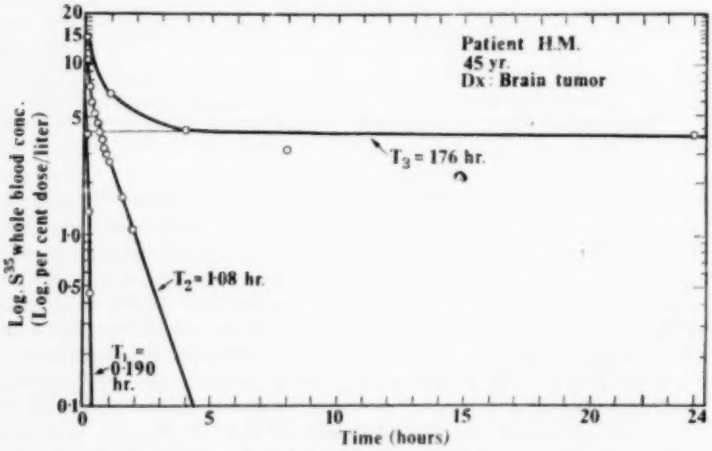

FIG. 3.—Resolution of the whole blood ^{35}S concentration curve obtained from a patient with a malignant neoplasm other than multiple myeloma into three exponential components. T_1, T_2 and T_3 are the corresponding initial, intermediate and late half-disappearance times.

^{35}S urinary excretion.—The ^{35}S excretion in the urine is relatively slow and about the same for both groups of patients (Table I). The output of the first day ranges from about 5 per cent of the dose to a maximum of about 20 per cent of the dose. From the second day on, the ^{35}S is eliminated in the urine exponentially the average doubling-time for cumulative excretion being about 7·2 days (Fig. 4) By the end of the first week, about 30 per cent of the dose has been excreted.

^{35}S-cystine spaces of dilution.—The zero-time radiosulfate whole blood spaces and their respective ratios to body surface and to weight are given in Table I. These results are similar for both the myeloma patients and those having other neoplasms. The average space for patients with myeloma is 7·39 $\pm$ 1·41 liters, corresponding to 13·2 $\pm$ 3·49 $\times$ 10^{-2} liter per kilogram of body weight. For the

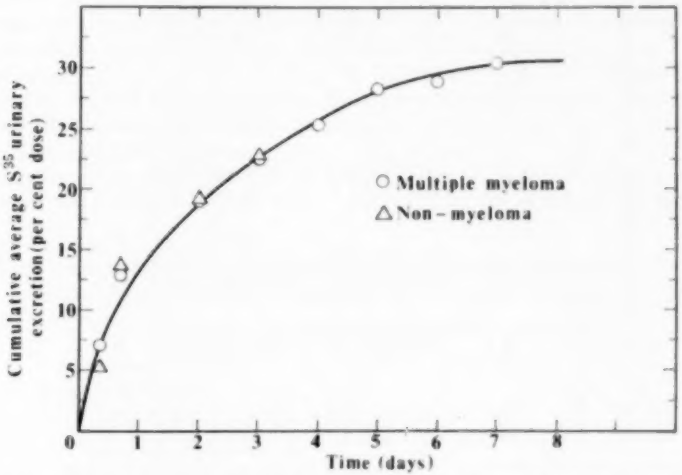

FIG. 4.—The cumulative mean ^{35}S urinary excretion for the first week following the administration of the tagged cystine.

other patients, an average space of 8·66 $\pm$ 2·67 liters or 14·7 $\pm$ 4·04 $\times$ 10^{-2} liter per kilogram of body weight was found. As was noted earlier, since the ^{35}S concentration in the whole blood and plasma are almost identical, the respective spaces are likewise very similar, and, for brevity, only the whole blood radiosulfate spaces are tabulated.

DISCUSSION

The intravenous administration of ^{35}S-l-cystine provides an immediate supply of this readily detected labeled amino acid to all sites of protein synthesis. The ^{35}S labeled cystine exchanges its labeled position with the other amino acids only slightly, if at all. The sulfur of ^{35}S-cystine is not transferred to methionine. It is believed that through the use of this amino acid the uptake and loss of isotope by the protein are almost exclusively in the form of the administered amino acid (Goldsworthy and Volwiler, 1957). The resulting turnover curve is usually divided into the following phases (Armstrong, Bronsky and Hershman, 1955; Golds-

TABLE I.—*^{35}S Urinary Excretion and Zero-time Whole Blood Spaces of Dilution*

Sex	Age	Diagnosis	% Urinary excretion: 1st day First 8 hrs	% Urinary excretion: 1st day Next 16 hrs	% Urinary excretion: 2nd day	% Urinary excretion: 3rd day	Space (liter)	Space / Body surface (liter/meter²)	Space / Wt. (liter/kg × 100)
		Group A							
♀	62	Multiple myeloma	8·30	6·30	5·65	4·44	9·75	6·17	16·6
♀	60	,,	5.64	8·50	7·93	2·96	6·50	3·65	9·05
♀	66	,,	2·38	2·86	6·30	2·09	7·50	4·78	13·4
♀	62	,,	6·44	6·76	5·18	3·88	7·60	6·23	17·6
♀	51	,,	12·5	5·03	5·42	4·59	5·62	3·53	9·51
		Average	7·05	5·89	6·10	3·59	7·39	4·87	13·2
			±3·33*	±1·88	±0·99	±0·94	±1·41	±1·17	±3·49
		Group B							
♂	57	Hypernephroma	3·15	4·50	4·64	4·21	7·50	4·57	13·1
♀	53	Carcinoma breast	6·96	10·3	5·83	3·28	10·2	5·63	14·1
♂	45	Brain tumour	0·00	12·7	7·09	5·57	6·50	3·87	10·9
♀	63	Papilloma breast	12·7	8·95	7·14	2·15	5·95	4·16	13·1
♀	75	Bronchogenic carcinoma	3·53	5·65	3·60	2·18	13·1	7·80	22·5
		Average	5·27	8·41	5·66	3·48	8·66	5·21	14·7
			±4·32	±2·99	±1·38	±1·30	±2·67	±1·58	±4·04

* Standard deviation.

worthy and Volwiler, 1957): (a) rapid incorporation of the amino acid radioactivity into the protein and rapid release of the newly synthesized protein into the circulation in the first 24 hours; (b) the distribution and mixing period, lasting a few days; (c) the exponential portion of the curve, presumed to reflect primarily the rate of metabolic degradation of the labeled protein; and (d), if the experiment continues for sufficient time, a phase reflecting the re-incorporation of the isotope into the protein.

In comparing the fate of tagged cystine with that of radiosulfate, some significant differences are observed. While the spaces of dilution for these two substances are quite similar and in accord with those reported for radiosulfate for normal subjects as well as for others having malignancies (Siegel *et al.*, 1959; Ryan *et al.*, 1956; Walser, Seldin and Grollman, 1953), the handling and turnover of these compounds are quite different. Fig. 1 illustrates the ^{35}S whole blood concentration as a function of time for those receiving labeled cystine. Following administration of labeled cystine the ^{35}S blood concentration decreases slowly; thus, by the second day, when the ^{35}S concentration following radiosulfate, as previously reported by us, is less than 0·5 per cent dose/liter, the ^{35}S concentration from cystine is about six times greater. This markedly slow fall in ^{35}S blood levels is characterized by the greatly prolonged late (T_3) half-disappearance times following labeled cystine (Table II) as compared to those found for radiosulfate.

TABLE II.—*Initial, Intermediate, and Late Half-disappearance Times obtained from ^{35}S Whole Blood and Plasma Concentration Curves.*

	Average half-disappearance times (hours)		
Patients	T_1	T_2	T_3
Multiple myeloma	0·448 ± 0·157*	— †	281 ± 77
Other neoplasms	0·265 ± 0·135	1·41 ± 0·65	162 ± 121

* Standard deviation.

† T_2 was found in two of the five multiple myeloma patients, averaging 2·47 hours.

Thus, the average value T_3 for patients with myeloma given ^{35}S-cystine is 281 hours as compared to 15·9 hours following tagged sulfate. A similar lengthening of this parameter is observed also for the patients with other neoplasms. There is a corresponding lower output of ^{35}S in the urine from ^{35}S-cystine as compared to that following administration of radiosulfate (Fig. 4). By the second day, when more than 60 per cent of the injected sulfate has been excreted, those given ^{35}S-cystine have excreted only about 20 per cent of the dose. Furthermore, for those given labeled cystine, an equilibrium is attained after the second day, the cumulative urinary output growing exponentially, with an average doubling-time, as noted earlier, of about 173 hours, which is in good agreement with the observed late half-disappearance times (Table II). Thus, after the second day, the constant fraction of the remaining dose, which disappears daily from the blood, was found in the urine, suggesting that the ^{35}S is then being degraded and directly excreted.

When ^{35}S-cystine is administered to control subjects, the label is incorporated into the normal serum albumin and globulins. When ^{35}S-cystine is given to patients with myeloma, the label also is incorporated into the normal albumin and globulins

as well as the abnormal myeloma globulins. The present investigation did not involve study of homologous serum proteins, and the curves obtained represent summation effects for all the serum protein complexes. It is possible that if individual globulins were studied, greater differences might have been observed between the turnover of the ^{35}S-cystine labeled normal globulins and the labeled myeloma globulins (Putnam, 1959).

There is a suggestive finding of a difference in the fate of ^{35}S-cystine for myeloma patients as compared to those having other malignancies. The initial (T_1) and late (T_3) half-disappearance times are longer for those having myeloma (Table II). This suggests that the hyperglobulinemia of myeloma may result from a slower catabolism of myeloma globulin. In this connection, Putnam and Hardy (1955) observed a greater half-disappearance time for the turnover of beta myeloma globulin than that obtained for the normal beta globulins by London (1950), and stated that the hyperglobulinemia of myeloma might be due to accumulation of the myeloma globulin by virtue of its slow turnover rate.

SUMMARY

^{35}S-l-cystine (specific activity 30 mc/g.) in doses of approximately 0·5 mc was administered intravenously to five patients with multiple myeloma and to five subjects with other neoplastic diseases. For both groups of patients, the ^{35}S concentration of whole blood and plasma were similar. The ^{35}S blood levels declined slowly; at five days after administration of the dose they were approximately three-quarters of the initial 8 hour values. The mean zero-time spaces of dilution were 7·39 $\pm$ 1·41 liters for the patients with multiple myeloma and 8·66 $\pm$ 2·67 liters for those with other neoplasms. ^{35}S urinary excretion was low, 13·6 to 30·9 per cent of the dose appearing in the urine within 72 hours. The turnover and excretion of ^{35}S administered as cystine are very much slower than when given as radiosulfate. By graphical analysis, it was possible to resolve the blood concentration curves into three exponential components. Patients with multiple myeloma were found to have longer initial and late half-disappearance times when compared to the subjects with other neoplastic diseases.

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REFERENCES

Armstrong, S. H., Jr., Bronsky, D. and Hershman, J.—(1955) *J. Lab. clin. Med.*, **46**, 857.

Goldsworthy, P. D. and Volwiler, W.—(1957) *Ann. N.Y. Acad. Sci.*, **70**, 26.

London, I. M.—(1950) The Robert Gould Research Foundation, Inc., Cincinnati, Symposium on Nutrition, **2**, 72.

Putnam, F. W.—(1959) *New Engl. J. Med.*, **261**, 902.

Idem and Hardy, S.—(1955) *J. biol. Chem.*, **212**, 371.

Ryan, R. J., Pascal, L. R., Inoye, T. and Bernstein, L.—(1956) *J. clin. Invest.*, **35**, 1119.

Sachs, B. A., Cady, P. and Ross, G.—(1954) *Amer. J. Med.*, **17**, 662.

Siegel, E., Sachs, B. A. and Graig, F. A.—(1959) *Proc. Soc. exp. Biol., N.Y.*, **101**, 53.

Walser, M., Seldin, D. W. and Grollman, A.—(1953) *J. clin. Invest.*, **32**, 299.

ACCUMULATION OF PHOSPHOETHANOLAMINE IN THE LIVERS OF RATS INJECTED WITH HEPATOCARCINOGENS

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THE addition of copper salts to a diet containing a carcinogenic azo dye affords a degree of protection against hepatocarcinogenesis to rats consuming the diet (Howell, 1958). It has also been found that rats which are fed 3′-methyl-4-dimethylaminoazobenzene (Neish, 1959*b*) or which receive single intraperitoneal injections of various hepatocarcinogens (Neish, 1958, 1959*a*) suffer a marked decline in the serum levels of total copper and of the copper-containing enzyme para-phenylenediamine oxidase (caeruloplasmin). The degree and persistence of this decline appear to be correlated closely with the carcinogenic potency of the injected materials. These results suggest that a derangement of copper metabolism in rat livers may occur at an early stage in hepatocarcinogenesis.

Serum levels of total copper and caeruloplasmin are generally subnormal in patients with hepatolenticular degeneration (Scheinberg and Sternlieb, 1959) and Uzman (1957) and Uzman *et al.* (1956) have detected an abnormal copper-avid ninhydrin-reacting substance, possibly a peptide, in the livers of such patients. It seemed important to determine whether hepatocarcinogens could induce the formation of similar abnormal peptides in rat liver. Although work on this aspect is not yet complete it has been found that injection of hepatocarcinogens into rats increases the levels of certain ninhydrin-positive materials in the liver.

Firstly, it was established qualitatively by paper chromatographic and electrophoretic investigations of phosphate or ethanol extracts of rat liver that intraperitoneal injections of hepatocarcinogens caused a marked increase in the level of "free" phosphoethanolamine (PE) in this tissue. In general, a strong hepatocarcinogen evoked and maintained a higher level of PE for a longer time than did a weaker one.

Secondly, it was found that the livers of rats injected with hepatocarcinogens contained one or two unidentified ninhydrin-positive substances X and Y which migrated towards the anode during paper electrophoresis at pH 8·6. Spot X was observed regularly after the application of a strong hepatocarcinogen and its intensity seemed to run parallel with the intensity of PE. Y was not always present when X occurred but it often appeared when X was rather intense. Traces of X but not of Y have been noted occasionally in extracts of normal rat liver. The nature of X and Y has not yet been completely elucidated but there is evidence that these substances are acidic peptides and that X at least seems to be closely related to glutathione.

In the present paper we shall discuss in detail the effect of hepatocarcinogens on the content of free PE in rat livers. In a future publication the occurrence and nature of the peptide-like materials X and Y will be considered in relation to similar substances which have been found in the livers of tumour-bearing rats and in tumour extracts.

EXPERIMENTAL

Adult male albino rats received single intraperitoneal injections of the azo dyes 3′-methyl-, 4′-methyl-, 2-methyl- and 4′-ethyl-4-dimethylaminoazobenzene at dose levels corresponding on a mole for mole basis with a standard injection of 16·5 mg. of 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB) in 0·6 ml. of arachis oil per 100 g. of body weight, as used in earlier work (Neish, 1958, 1959*a*) on copper metabolism. At this dose level, 2-methyl-4-dimethylaminoazobenzene (2-MeDAB) proved to be rather toxic and about half of the animals died within 2 days of the injection. Other rats received intraperitoneal injections of the hepatocarcinogens tannic acid (5 mg./100 g. body weight (b.w.)), dimethylnitrosamine (2·5 mg./100 g. b.w.), DL-ethionine (11·3 mg./100 g. b.w.), and of the supposedly non-carcinogenic carbon tetrachloride (50 mg./100 g. b.w.) suspended or dissolved in arachis oil (0·6 ml./100 g. b.w.). Control rats received injections of arachis oil only (0·6 ml./100 g. b.w.).

At intervals after injections, control and experimental animals were killed with ether, bled from the heart and the livers perfused via the portal vein with chilled normal saline. The livers were removed, weighed and stored at once at $\sim -15°$ C. Not later than 2 hours after perfusion, 2 g. portions of the frozen livers were homogenized with 4 ml. of full strength Sorensen phosphate buffer at pH 7·2 in an all-glass homogenizer cooled in ice-water. Supernatants were obtained by centrifugation of the homogenates for 2 hours at approximately 4500 r.p.m. and 40° F. in an International Portable Refrigerating Centrifuge Model PR–1. The supernatants were stored at $-15°$ C. and 0·01 ml. aliquots were used as required for paper chromatographic and electrophoretic studies. These extracts seemed to be stable indefinitely if stored at $-15°$ C.

Alcoholic extracts of livers were prepared by homogenization of 1 g. portions of the frozen livers with sufficient ice-cold ethanol to give a final supernatant containing approximately 80 per cent alcohol. In order to assess the amount of absolute ethanol to be added, liver dry weights were determined by drying 1 g. portions of the livers over sulphuric acid *in vacuo* at $+4°$ C. to constant weight. The alcoholic homogenates were centrifuged for 10 minutes at 2000 r.p.m. and 0·01 ml. aliquots of the supernatants were used for paper chromatographic studies.

Two dimensional paper chromatography.—Separations were carried out on 20 cm. squares of Whatman No. 1 paper in the solvent systems (ascending) recommended by Bowden (1959): in the first direction, 80 per cent phenol with an ammonia atmosphere (sodium cyanide was not included in the tank) and in the second direction, a mixture of *n*-butanol (100 ml.), methyl ethyl ketone (100 ml.), dicyclohexylamine (20 ml.) and water (47 ml.). An aliquot (0·01 ml.) of liver extract was placed on the paper at a point 3 cm. from adjacent edges.

In early experiments, the papers were stitched with thread in the form of cylinders which were allowed to stand in $\sim$ 80 ml. of developing solvent in litre beakers in an enclosed glass tank. Later, a duralumin Datta frame (Bowden, 1959) was used for multiple separations but this proved unsatisfactory in the phenol run owing to serious contamination of the papers with coloured products which apparently arose through the action of phenol on the metal frame. A modified Datta frame which functioned satisfactorily was constructed as follows: two end pieces, H-shaped, were made of glass rods of suitable length and thickness

connected with a pair of brass X blocks ; at each arm of the H an X block was placed and 4 narrow glass rods were supported by these X blocks to form a frame. A dozen chromatography papers (20 × 20 cm.) with corner holes, as supplied for use with the Datta frame, were placed on the glass frame and separated from one another by pairs of porcelain penicillin cups. After drying, the papers were treated with a solution of ninhydrin (0·2 per cent w/v) in *n*-butanol to 100 ml. of which was added 4 ml. of glacial acetic acid, dried at room temperature and heated for 10 minutes at 70° C. Permanent records of ninhydrin-positive spots were made with auto-positive document paper No. 43.

Phosphate buffer salts did not markedly disturb the separation of pure amino acid mixtures in this chromatographic system but protein or polypeptide constituents of liver phosphate extracts seemed to interfere with the separation of substances with high Rf values in phenol. More sharply defined chromatograms were obtained when alcoholic extracts of liver were used and this method proved most useful for comparative studies of the PE content of livers.

Paper electrophoresis.—Usually 4 × 0·01 ml. aliquots of liver phosphate extracts were applied at the mid-line of a strip of Whatman No. 1 paper (12 × 50 cm.) previously moistened with pH 8·6 barbitone buffer (600 ml. of a solution of sodium barbitone (10·3 g./litre) plus 400 ml. of a solution of barbitone (1·85 g./litre) and blotted. The paper was supported as an inverted V by a glass rod under the mid-line in a modification of Durrum's apparatus (Durrum, 1950). The ends of the paper dipped into glass tanks containing ~ 900 ml. of pH 8·6 buffer. Electrical connection was established by KCl-agar bridges (3 per cent agar agar in saturated KCl) connecting the main buffer compartments to small glass compartments filled with buffer and provided with carbon electrodes. Current was supplied by 3 × 120 volt high tension batteries in series. After 3 hours, the paper was removed, dried, and ninhydrin-reacting spots were revealed by spraying with the ninhydrin-acetic acid mixture mentioned above. The papers were then dried in an oven at 120° C. until the spot due to aspartic acid which had initially a distinctive blue colour began to turn reddish (~ 5 minutes). By this time spots X and Y if present appeared. In earlier work, the papers were sprayed with a solution of ninhydrin (0·2 per cent w/v) in *n*-butanol containing no acetic acid. Under these circumstances X and Y usually became clearly discernible only on the day following spraying and heating.

Further analysis of ninhydrin-positive spots was carried out on sections cut from electropherograms run simultaneously. Ninhydrin-positive materials were eluted from the sections with deionized water. The aqueous extracts were freeze-dried, the residues taken into a small volume of deionized water and the solutions subjected to two dimensional chromatography as described above. Satisfactory separations were obtained with apparently little or no interference from the barbitone components present in the extracts. Likewise mixtures of amino acids dissolved in barbitone buffer separated well in Bowden's system.

RESULTS

The accompanying figures illustrate the accumulation of phosphoethanolamine and of substances X and Y in the livers of rats injected with hepatocarcinogens.

Fig. 1 shows two dimensional chromatograms (cylinder method) of phosphate extracts of livers from rats injected with (*A*) arachis oil only, (*B*) 3′-MeDAB and

(*C*) 4′-EtDAB. The livers were obtained 6 days after injection. According to Miller, Miller and Finger (1957) 3′-MeDAB and 4′-EtDAB are equally potent carcinogens. Each substance caused about the same degree of accumulation of PE in rat liver. When synthetic PE was added to the normal liver extract, the chromatogram showed an increase in the intensity of the rather weak spot due to naturally occurring PE.

Two dimensional chromatograms (frame method) of phosphate extracts of livers from rats (*A*) 3 days and (*B*) 6 days after carbon tetrachloride injection are shown in Fig. 2. A reference chromatogram (*C*) is included to show the separation of PE from a synthetic mixture of several of the amino acids regularly encountered in the liver extracts. Note the higher level of PE in the 3-day CCl_4 liver

EXPLANATION OF PLATES

List of abbreviations for ninhydrin-positive spots:

PE = phosphoethanolamine.
GLU = glutamic acid.
GLY = glycine.
ASP = aspartic acid.
TAU = taurine.
G = glutathione.

Vertical and horizontal arrows intersecting at the origin of 2-dimensional chromatograms indicate the direction of flow of aqueous phenol and of butanol-methylethylketone-dicyclohexylamine-water solvents respectively.

FIG. 1.—Two-dimensional chromatograms of phosphate extracts of livers from rats 6 days after intraperitoneal injection of

A — arachis oil only.
B — 3′-MeDAB in arachis oil.
C — 4′-EtDAB in arachis oil.

FIG. 2.—Two-dimensional chromatograms of phosphate extracts of rat liver.

A—3 days after carbon tetrachloride injection.
B—6 days after carbon tetrachloride injection.

C is a reference chromatogram showing separation of a mixture of the indicated amino acids from 0·005 ml. of a solution containing 1 mg. of each substance in 5 ml. of phosphate buffer pH 7·2. Each spot equivalent to 1 μg.
Note the double spot due to glutathione.

FIG. 3.—Electropherograms (Whatman No. 1 paper, pH 8·6 barbitone, 360 volts, 3 hours) of phosphate extracts of

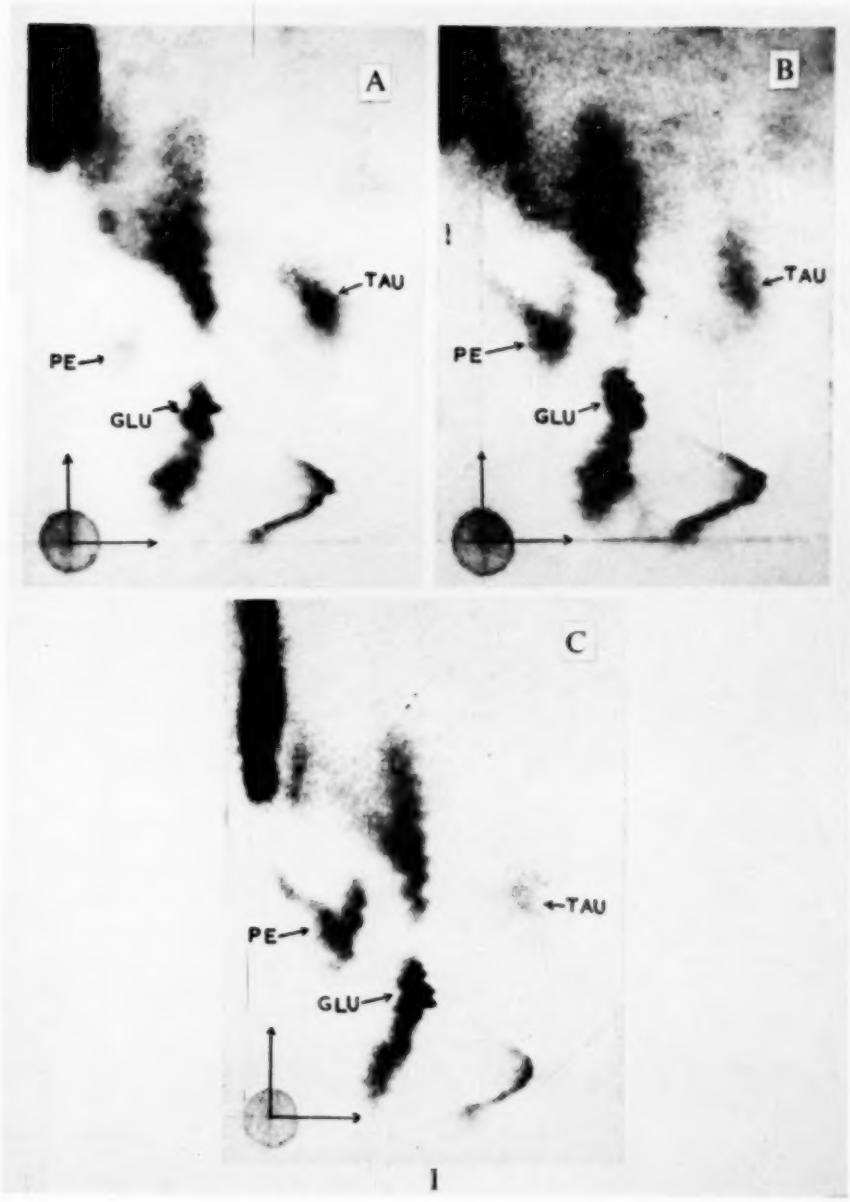
A — rat liver 3 days after tannic acid injection.
B — rat liver 3 days after arachis oil injection.
C = 2-dimensional chromatogram of spot 4 from electropherogram A.
D = 2-dimensional chromatogram of spot 4 from electropherogram B.

Electropherograms (as above, 2 hour run) of phosphate extracts of

E — rat liver 6 days after 3′-MeDAB injection.
F — rat liver 6 days after 2-MeDAB injection.
G — rat liver 6 days after injection of arachis oil only.

FIG. 4.—Two dimensional chromatograms of ethanolic extracts of rat livers obtained 3 days after injection of

A — arachis oil only.
B — 3′-MeDAB.
C — 4′-MeDAB.
D — 2-MeDAB.



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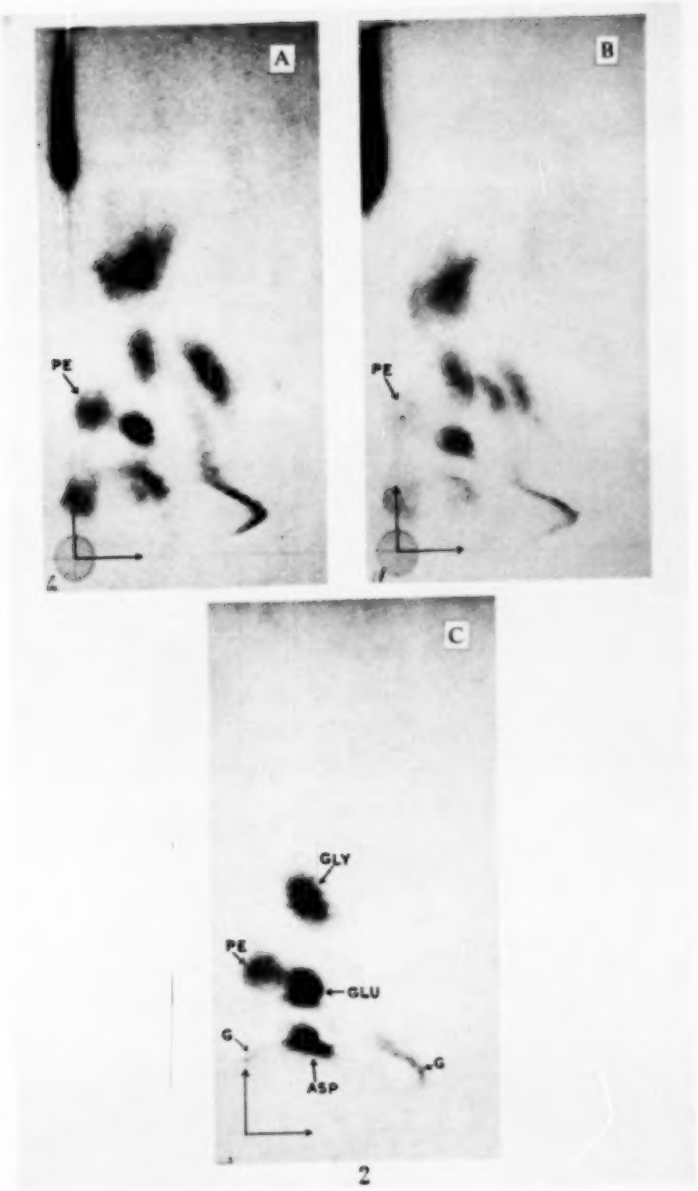

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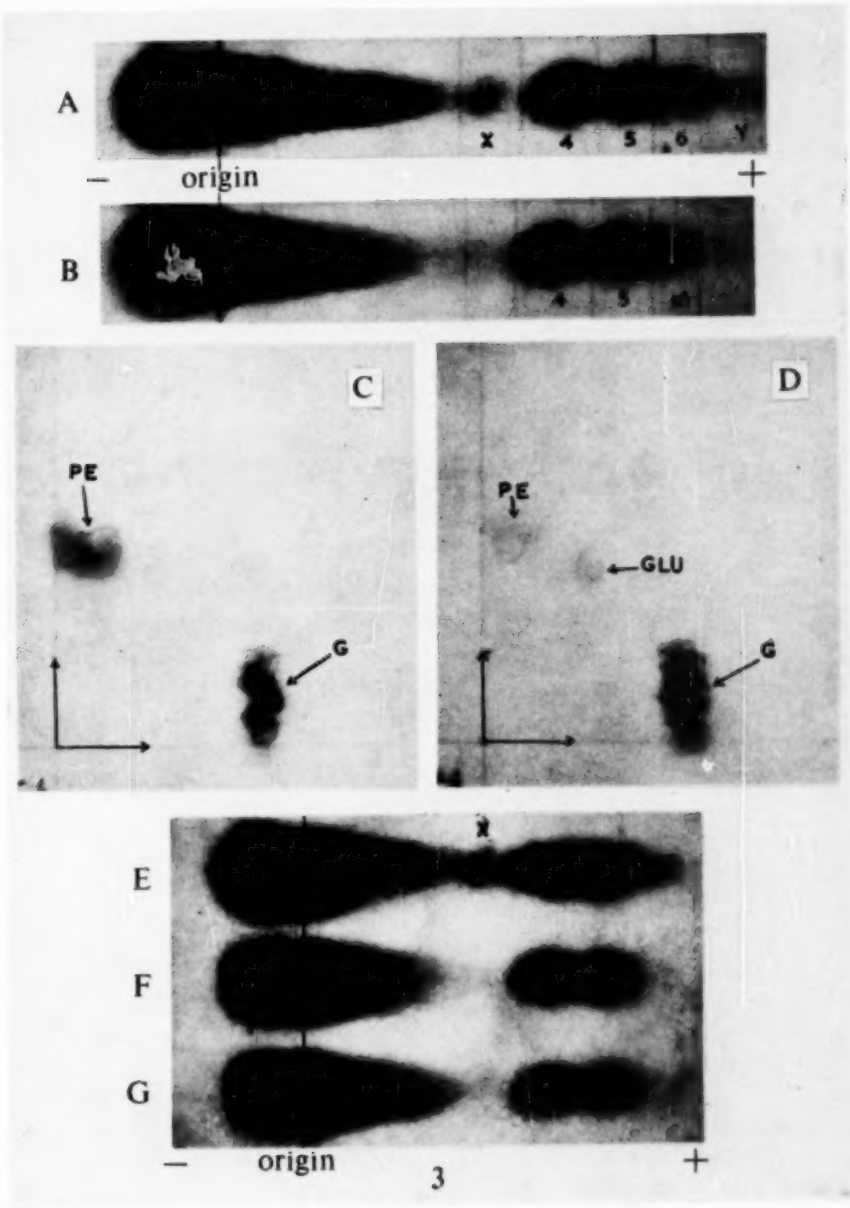

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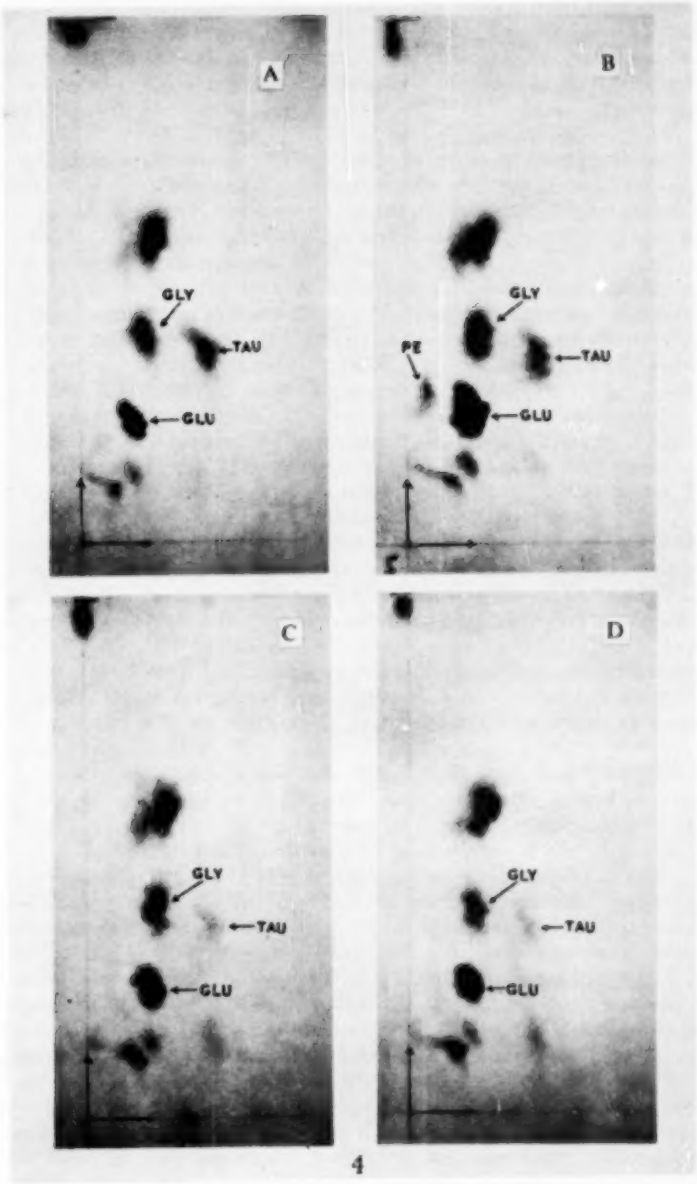

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as compared with the 6-day CCl_4 liver. The latter chromatogram (*B*) is practically identical with those given by extracts of a 10-day CCl_4 liver and of a normal liver. Although carbon tetrachloride is said to be non-carcinogenic for rat liver, it resembles the feeble carcinogen 4′-MeDAB in that both substances have a small capacity for increasing the " free " P.E. content of rat liver 3 days after injection.

Electrophoretic separations of phosphate extracts of a normal rat liver (*B*) and of the liver of a rat injected 3 days previously with tannic acid (*A*) are shown in Fig. 3. Spots X and Y are present in the tannic acid liver extract but not in the normal liver. Spots marked 4, 5 and 6 were shown to consist of PE + glutathione, glutamic and aspartic acids respectively.

Paper strips corresponding to spot 4 for normal and tannic acid liver separations were cut from 3 parallel separations for each extract and the eluted ninhydrin-positive materials were subjected to two-dimensional chromatography. Fig. 3 (*C*) shows the marked accumulation of PE in the liver of the rat injected with tannic acid as compared with the normal rat liver (*D*). Fig. 3 (*E*, *F* and *G*) are electropherograms showing the occurrence of X in a rat liver (*E*) 6 days after injection with the powerful carcinogen 3′-MeDAB. Spot X is not present in the liver of a normal rat (*G*) or in the liver of a rat 6 days after injection of the non-carcinogen 2-MeDAB (*F*). The nature of ninhydrin-positive spots X and Y will be discussed in a separate communication.

The use of ethanolic extracts of livers for demonstrating accumulation of PE due to hepatocarcinogen treatment is shown in Fig. 4. Livers were obtained 3 days after injection from rats which received (*A*) arachis oil only, (*B*) 3′-MeDAB (carcinogenic), (*C*) 4′-MeDAB (weakly carcinogenic) and (*D*) 2-MeDAB (non-carcinogenic). Note the accumulation of PE due to 3′-MeDAB. Studies of phosphate extracts of the same livers revealed that there was a slight accumulation of PE due to the feebly carcinogenic 4′-MeDAB, 3 days after injection, but only normal low levels of PE were observed at 6, 10 and 18 days after injection of this substance (Table I).

In the course of this work it has been found that there is a transient decrease in the dry weight of rat livers following a single intraperitoneal injection of a powerful hepatocarcinogen. From the results for five experiments collected in Table I it is apparent that the degree and persistence of the decrease in liver dry weight is especially marked in the livers of rats injected with the most powerful carcinogens, namely 3′-MeDAB, 4′-EtDAB, tannic acid and dimethylnitrosamine. These results confirm and extend the work of Sauberlich and Baumann (1951) who noted that the feeding of carcinogenic but not of non-carcinogenic azo dyes produced a decrease in the dry weight of rat liver. In general, we find that the elevated levels of PE and the appearance of X parallel this decrease in liver dry weight percentage. It is of interest to note that after injection of powerful hepatocarcinogens, there is usually an appreciable diminution in the weight of the whole liver expressed as a percentage of the total body weight. In Table I (experiment V) we give these values for the livers of normal rats, for rats treated with the carcinogenic 3′-MeDAB, and for rats injected with the non-carcinogenic 2-MeDAB. Note the hepatomegaly due to the last mentioned substance.

In Table II are shown the relative PE contents of ethanolic extracts of rat liver at various times after injection of carcinogenic or non-carcinogenic substances.

TABLE I.—*Effect of Various Hepatocarcinogens and Related Substances on the Dry Weight Percentage of Rat Liver*

Substance injected in arachis oil	Time after injection (days)	Liver dry weight percentage in experiment No.				
		I	II	III	IV	V
Nil	3	26·6	26·1	29·2	30·0	29·1 (4·8)*
	6	26·8	25·4	27·5	28·6	27·2 (4·6)
	10	26·3	—	26·3	31·6	—
	18	26·7	—	—	—	—
3′–MeDAB	3	22·2	23·8	—	—	22·4 (2·9)
	6	22·7	24·1	—	—	21·8 (2·6)
	10	26·6	—	—	—	—
	18	26·9	—	—	—	—
4′–MeDAB	3	29·0	—	—	—	—
	6	26·1	—	—	—	—
	10	27·6	—	—	—	—
	18	27·4	—	—	—	—
2–MeDAB	3	24·9	—	—	—	28·5 (5·1)
	6	—	—	—	—	25·9 (5·7)
	10	27·4	—	—	—	—
4′–EtDAB	3	—	23·7	—	—	—
	6	—	20·9	—	—	—
Tannic acid	3	—	—	26·9	—	—
	6	—	—	22·9	—	—
	12	—	—	26·7	—	—
DL-ethionine	3	—	—	29·9	—	—
	6	—	—	27·2	—	—
	12	—	—	24·3	—	—
Dimethylnitrosamine	3	—	—	23·0	25·7	—
	6	—	—	—	28·1	—
	12	—	—	—	27·4	—
Carbon tetrachloride	3	—	—	—	28·6	—
	6	—	—	—	32·4	—
	12	—	—	—	29·1	—

* Figures in parentheses, liver as percentage of total body weight.

It is interesting that marked accumulation of PE occurred in the case of DL-ethionine only at the 12th day after injection at which time the decrease in liver dry weight percentage was most marked.

Although elevations of the level of PE is the most prominent feature in chromatograms of extracts of carcinogen-treated rat livers, changes in the levels of other ninhydrin-positive substances do occur. For example, 3 days after injection of the azo dyes (Fig. 4) the following changes were observed :

Substance injected in arachis oil	PE	Levels of glutamic acid	Glycine	Taurine
Nil	−	++	+	+++
3′–MeDAB	+	++++	++	+++
4′–MeDAB	−	++	+	+
2–MeDAB	−	++	+	+

TABLE II.—*Relative PE Contents of Rat Liver at Various Times After Injection of Carcinogenic or Non-carcinogenic Substances*

Substance injected in arachis oil	Carcinogenicity	Level of phosphoethanolamine after injection* (at days)			
		3	6	10–12	18
3′-MeDAB	+ + −	+ +	+ +	−	−
4′-EtDAB	+ + −	+ +	+ +	−	−
4′-MeDAB	+	±	−	−	−
2-MeDAB	−	−	−	−	
Tannic acid	+ + +	+	+ +	+	
Dimethylnitrosamine	+ + +	+	+ +	+	
DL-ethionine	+ +	−	−	+	
Carbon tetrachloride	−	±	−	−	
Nil	−	−	−	−	−

* When no symbol is shown, no experiment was performed at the stated time. For the assessment of PE spot intensity, scoring was done visually. At least two livers were examined for each injection time.

Normal livers do in fact contain PE but under our conditions of alcoholic extraction it is barely detectable chromatographically in the extracts and the level is rate −. Under the influence of powerful hepatocarcinogens, in spite of the decline in liver dry weight percentage, prominent PE spots are observed in extracts of these livers. In these instances, the liver PE content evidently greatly exceeds the normal level which according to Awapara, Landua and Fuerst (1950) is of the order of 17 mg./100 g. wet weight of liver.

As compared with normal liver, elevated levels of PE, glutamic acid and glycine occurred 3 days after injection of 3′-MeDAB. On the other hand 4′-MeDAB and 2-MeDAB did not alter the levels of these substances, but the taurine content of the livers was appreciably reduced. This suggests that cysteine which is probably the precursor of taurine is being utilized in some detoxication reaction. At 6 days after injection of 3′-MeDAB, the liver had an elevated level of PE and of glutamic acid as compared with a control liver, but the glycine level was then normal and that of taurine much reduced. At later times the glutamic acid level of 3′-MeDAB livers returned to normal.

DISCUSSION

The accumulation of PE in the livers of rats injected with hepatocarcinogens cannot yet be explained. Perhaps it is due to breakdown of phosphatidylethanolamine (cephalin) which might accompany the loss of dry matter in rat liver exposed to hepatocarcinogens or it might be the consequence of an impairment in cephalin synthesis. The first possibility seems unlikely because there is no evidence yet that enzymes capable of liberating PE from phosphatidylethanolamine are present in mammalian tissues. With regard to the second possibility, Kennedy and Weiss (1956) showed that cytidine triphosphate reacts with PE in the presence of a transferase to yield the nucleotide cytidine diphosphate ethanolamine which in turn under the influence of another enzyme combines with a lipid acceptor to form phosphatidylethanolamine. In the absence of cytidine triphosphate, PE might accumulate. The observation by Reid and Lotz (1958) that there is a marked accumulation of uridine-5′-phosphate in the livers of rats fed 3′-MeDAB seems to suggest that pathways from uridine to cytidine triphosphate might indeed be blocked by the hepatocarcinogen.

Another possible explanation for PE accumulation may be found in the suppression of liver alkaline phosphatase activity by hepatocarcinogens. According to McCance, Morrison and Dent (1955) and to Fraser, Yendt and Christie (1955) patients with hypophosphatasia excrete PE in their urine. This is attributed not to renal malfunction but to a lowered serum alkaline phosphatase activity resulting in accumulation and excretion of PE. According to McCance *et al.* (1955), PE can act as a substrate for alkaline phosphatase. Sachs, Bäumer and Menkhaus (1959) found by histochemical methods that oral administration of the hepatocarcinogen thioacetamide to rats does indeed lead to a decrease in liver alkaline phosphatase and Tsuboi and Stowell (1951) observed a decline in the alkaline phosphatase activity of mouse liver following a single feeding of carbon tetrachloride (carcinogenic for mouse liver). Woodard (1943), however, found a marked elevation of alkaline phosphatase in the precancerous livers of rats fed 4-dimethylaminoazobenzene.

Since serine is a probable precursor of ethanolamine and PE it was unfortunate that in the present study certain limitations of the chromatographic technique prevented us from following easily the fate of serine and ethanolamine in rat liver subjected to the action of hepatocarcinogens. It is hoped to complete this aspect of the work. Meanwhile it may be noted that Levy, Montanez and Dunn (1955) reported a decrease in the serine level in rat liver after a single massive intraperitoneal injection of the hepatocarcinogen, DL-ethionine.

Kensler, Bierman and Condouris (1955) reported that the addition of ethanalamine to a diet containing 4-dimethylaminoazobenzene afforded marked protection to rats against hepatocarcinogenesis. In view of our findings it would seem that ethanolamine supplements can overcome some deleterious action of a hepatocarcinogen which is expressed in the accumulation of PE in the liver.

In certain other aspects of liver cancer, peculiarities in ethanolamine metabolism have been noted. Thus Reid, Landefetd and Simpson (1952) have shown that the ethanolamine moiety of the choline of rat liver tumours is apparently synthesized in a different way from that of normal rat liver. Dent, Fowler and Walshe (1951) have observed the excretion of large amounts of ethanolamine by a patient with primary hepatoma. This metabolic defect was considered to be either cause or consequence of the hepatoma.

It is of interest to recall the statement by Haven and Bloor (1956) that "abnormalities in the metabolism of ethanolamine if established as directly concerned in carcinogenesis by azo dyes would implicate phospholipids in the induction of hepatoma." Because of the close correlation which has been observed between the hepatocarcinogenic potency of a substance and its ability to cause accumulation of PE in rat liver, a more detailed biochemical study certainly seems warranted of the metabolism of phospholipids, serine, ethanolamine and PE in the stages of hepatocarcinogensis.

SUMMARY

Single intraperitoneal injections of powerful hepatocarcinogens into male albino rats caused a marked accumulation of phosphoethanolamine in the livers and the appearance of an unknown ninhydrin-positive substance having the properties of an acidic thiol peptide.

ADDENDUM

Since writing this our attention has been directed to several references which may have a bearing on our investigations.

(*a*) Ferrari and Tenconi (1957) stated that the livers of normal and adrenalectomized rats which had been injected repeatedly with 10 per cent ethanol contained respectively 8·0 and 23·6 mg. of free PE per 100 g. of fresh tissue.

(*b*) An increase in the free PE content of the livers of rats subjected to adrenalectomy had also been noted by Awapara, Skellenger and Manz (1955).

(*c*) Spicer and Weise (1956) observed an elevation of PE in the livers of rabbits and guinea-pigs which had been exposed to X-irradiation.

With regard to (*a*) and (*b*) it may be noted that DaVanzo and Eversole (1958) and Symeonidis, Mulay and Burgoyne (1954) found that total adrenalectomy protects rats against azo dye carcinogenesis. Perhaps the transient rise in liver PE following injection of an hepatocarcinogen is to be explained by some temporary deleterious action of the carcinogen on the adrenals.

REFERENCES

Awapara, J., Landua, A. J. and Fuerst, R.—(1950) *J. biol. Chem.*, **183**, 545.

Idem, Skellenger, W. and Manz, N.—(1955) *Tex. Rep. Biol. Med.*, **13**, 1.

Bowden, C. H.—(1959) *Clin. Chim. Acta*, **4**, 539.

Davanzo, J. P. and Eversole, W. J.—(1958) *Cancer Res.*, **18**, 796.

Dent, C. E., Fowler, D. I. and Walshe, J. M.—(1951) *Biochem. J.*, **48**, xiii.

Durrum, E. L.—(1950) *J. Amer. chem. Soc.*, **72**, 2943.

Ferrari, V. and Tenconi, L. T.—(1957) *G. Biochim.*, **6**, 137.

Fraser, D., Yendt, E. R. and Christie, F. H. E.—(1955) *Lancet*, i, 286.

Haven, F. L. and Bloor, W. R.—(1956) *Advanc. Cancer Res.*, **4**, 238.

Howell, J. S.—(1958) *Brit. J. Cancer*, **12**, 594.

Kennedy, E. P. and Weiss, S. B.—(1956) *J. biol. Chem.*, **222**, 193.

Kensler, C. J., Bierman, E. and Condouris, G.—(1955) *J. nat. Cancer Inst.*, **15**, 1569.

Levy, H. M., Montanez, G. and Dunn, M. S.—(1955) *J. biol. Chem.*, **212**, 985.

McCance, R. A., Morrison, A. B. and Dent, C. E.—(1955) *Lancet*, i, 131.

Miller, J. A., Miller, E. C. and Finger, G. C.—(1957) *Cancer Res.* **17**, 387.

Neish, W. J. P.—(1958) *Experientia*, **14**, 287.—(1959*a*) *Ibid.*, **15**, 20.—(1959*b*) *Ibid.*, **15**, 336.

Reid, E. and Lotz, F.—(1958) *Brit. J. Cancer*, **12**, 419.

Reid, J. C., Landefeld, M. O. and Simpson, J. L.—(1952) *J. nat. Cancer Inst.*, **12**, 929.

Sachs, H. W., Bäumer, A. and Menkhaus, G.—(1959) *Acta Hepato-Splenol.*, **6**, 286.

Sauberlich, H. E. and Baumann, C. A.—(1951) *Cancer Res.* **11**, 67.

Scheinberg, I. H. and Sternleib, I.—(1959) *Gastroenterology*, **37**, 550.

Spicer, S. S. and Weise, V.—(1956) *Enzymologia*, **17**, 263.

Symeonidis, A., Mulay, A. S. and Burgoyne, F. H.—(1954) *J. nat. Cancer Inst.*, **14**, 805.

Tsuboi, K. K. and Stowell, R. E.—(1951) *Cancer Res.* **11**, 221.

Uzman, L. L.—(1957) *Arch. Path.*, **64**, 464.

Idem, Iber, F. L., Chalmers, T. C. and Knowlton, M.—(1956) *Amer. J. med. Sci.*, **231**, 511.

Woodard, H. Q.—(1943) *Cancer Res.* **3**, 159.

THE EFFECTS OF SOME CHEMICAL CARCINOGENS UPON THE –SH LEVELS OF TARGET AND NON-TARGET TISSUES

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THE role of sulphydryl (–SH) groups in both tumour growth and the induction of tumours has been a subject of interest for many years. Actual experimental work in this field has been limited and often only via indirect approaches. Methods of measuring the –SH within tissues have been unsatisfactory and in many cases involved tedious procedures with specialised equipment. Recently, Calcutt and Doxey (1959) described a simple technique for tissue –SH measurements using small weights of tissue. This technique was then used to determine the effects of various carcinogenic and non-carcinogenic hydrocarbons on the liver –SH values of mice previously treated with one of these hydrocarbons (Calcutt, Doxey and Coates, 1959). This work dealt with the relationships of –SH levels to the metabolism of the agents in question, since all are known to be metabolised in the liver but none can be regarded as a true hepato-carcinogen.

The present paper records a study of the effects of known chemical carcinogens on the –SH levels of susceptible and non-susceptible tissues. We have been influenced in this choice of subject by knowledge of two previous papers which bear upon this field. Boyland and Mawson (1938) found that intraperitoneal injection of 3 : 4 : 5 : 6 dibenzocarbazole (a known hepato-carcinogen) causes a considerable rise in liver glutathione which is persistent over several months. The livers of mice injected with methylcholanthrene or 1 : 2 : 5 : 6 dibenzanthracene (neither being a liver carcinogen) did not show a similar increase in glutathione. Di Paolo and Niedbala (1957) found that after a single painting with either 1 : 2 : 5 : 6 dibenzanthracene or 9 : 10 dimethylbenzanthracene the –SH content of mouse skin was raised above the normal value and this increase was persistent for at least five days. The chemically related, but non-carcinogenic compound, anthracene, was found to have no effect on skin –SH levels.

The Measurement of Tissue –SH

As the technique used has been described in detail elsewhere, only an outline will be given here. Known weights of slices of the tissue under examination are immersed in a known volume of a standardised 5×10^{-5} M solution of *p*-chloromercuribenzoic acid (C.M.B.). After a time interval (approximately 30 minutes) for reaction with the –SH groups to occur, the unchanged C.M.B. is titrated potentiometrically with 2×10^{-4} M cysteine hydrochloride. The millivoltmeters originally used in developing this technique have now been replaced by transistorised titrimeters based on the design of Stock (1958). These have been found superior to millivoltmeters in use as settling times are negligible and end point

deflections are large and easily read. During the present work every titration has been run in duplicate, the two runs being done at the same time on separate sets of instruments. End points on the two sets of equipment have been either completely in agreement or within 0·1 ml. of one another. The mean of the two results has been used for calculation purposes. The technique has been adapted for glutathione estimations. Weighed amounts of tissue have been ground in a few ml. of 10 per cent metaphosphoric acid and centrifuged. The supernatant together with a further washing of the tissue has then been neutralised to pH 7·0 with 5 per cent caustic soda solution using Bromo-cresol purple as an indicator. This solution has been treated for –SH measurement as in the case of tissues. The neutralisation of the acid solution of glutathione is essential as C.M.B. is insoluble in acids.

The weights of tissue used for estimations have normally been in the range 80–200 mg.

Experimental

3 : 4 *Benzopyrene.*—One hundred and seventy strong A male mice were divided into two groups. The animals of one group each received a once weekly application of 0·2 ml. of a 0·5 per cent acetone solution of 3 : 4 benzopyrene. The other group were used as controls. Daily for the first week and then at intervals of seven days five animals of each group were killed. Small pieces of skin (after the fur had been clipped) were removed from the painted areas or from the corresponding areas of the untreated mice. Estimations of –SH were then made on the pooled samples of skin. The results over a period of 65 days are shown in Fig. 1. Since, over this period, no pattern has been detectable in the levels for the control skins these figures have been shown as a mean figure and a standard deviation. All measurements are expressed in terms of μg. of –SH per 100 mg. of wet weight of skin.

After the first treatment with benzopyrene the –SH level of the skin rose and then declined over the first week to a normal level. Further treatment gave enhanced levels for about 6–7 weeks when a return to the normal level occurred.

It has been shown by Calcutt and Powell (1947) that after skin painting of polycyclic hydrocarbons a large amount of the applied reagent is removed by the animals licking themselves. The agent removed in this fashion is carried down the gut and is transferred to the internal organs of the body. We have, therefore, done –SH measurements on both the liver and the kidneys of both control and experimental animals. The choice of these two tissues is based on the evidence of Weigert and Mottram (1946) that benzopyrene is metabolised in both, but that there is no good evidence for either being susceptible to the carcinogenic activity of 3 : 4 benzopyrene.

In Fig. 2 the results obtained with liver are shown. No definite indications of any well defined changes were obtained. The kidney results are shown in Fig. 3. Here again there was no evidence of specific changes in –SH levels.

N–2 Fluoroenylacetamide (*2 acetylaminofluorene ; 2.A.A.F.*).—Fifty female Wistar rats aged twelve weeks were divided into two equal groups. Both groups were fed a diet prepared by grinding up a normal pelletted food and making this up with water to give a firm mash. The carcinogen, 2.A.A.F., was added at the rate of 0·4 per cent to the diet of one group. Food prepared in this fashion was found to be acceptable to the animals and the control group ate and grew as

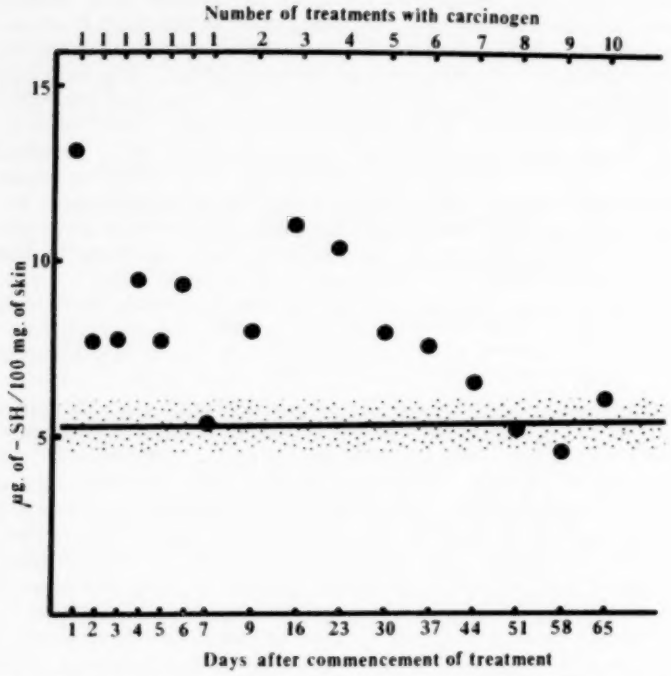
Number of treatments with carcinogen
1 1 1 1 1 1 1 2 3 4 5 6 7 8 9 10
15
10
5
μg. of – SH/100 mg. of skin
1 2 3 4 5 6 7 9 16 23 30 37 44 51 58 65
Days after commencement of treatment

FIG. 1.—The effects of treatment with 3 : 4 benzopyrene on mouse skin –SH levels.
In this and all further figures, the mean control value is shown as a heavy line and the standard deviation by the dotted area. Experimental figures are shown as filled circles.

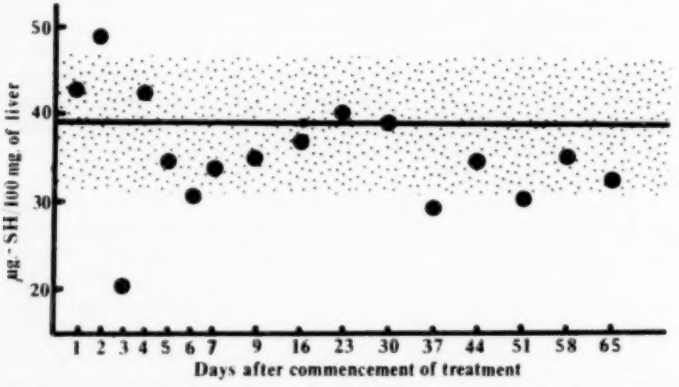

FIG. 2.—Mouse liver –SH values from animals which had been painted with 3 : 4 benzopyrene.

normally. The experimental group were found to eat slightly less than the controls.

At weekly intervals one animal from each group was killed and liver –SH levels determined. The results are shown in Fig. 4. As no pattern was found

FIG. 3.—Mouse kidney –SH levels from animals which had been painted with 3 : 4 benzopyrene.

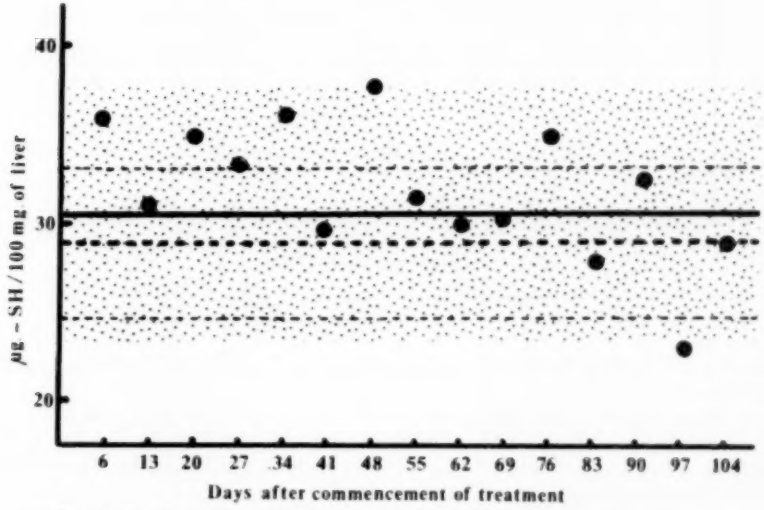

FIG. 4.—The effects of feeding 2.A.A.F. on rat liver –SH levels.
Heavy dash line represents amended mean control value and the limits of the standard deviation are shown as a lighter dash line (for details see text).

in the control levels a mean and standard deviation have been calculated and the experimental results are shown against these figures. The control figures contained two exceptionally high values and these have made the mean figure and the standard deviation much higher than would otherwise be the case. Recalculation of a mean and standard deviation, omitting these two very high

values, gave lower figures which were in close agreement with another set of figures independently determined upon a similar group of rats for another purpose. The amended mean and standard deviation have also been shown on Fig. 4. Whichever figure is taken as representative of the mean control value the results indicate an elevation of liver –SH over the first 7–8 weeks of feeding the carcinogen. Values then return to near the control value.

Hartwell (1951) and Shubik and Hartwell (1957) in their surveys of agents tested for carcinogenicity list several references to the induction of kidney tumours by 2.A.A.F. We have, therefore, also examined the kidney –SH levels from the same rats as were used in the previous experiment. The results are shown in Fig. 5. The picture, although not well defined, is suggestive of a rise in kidney

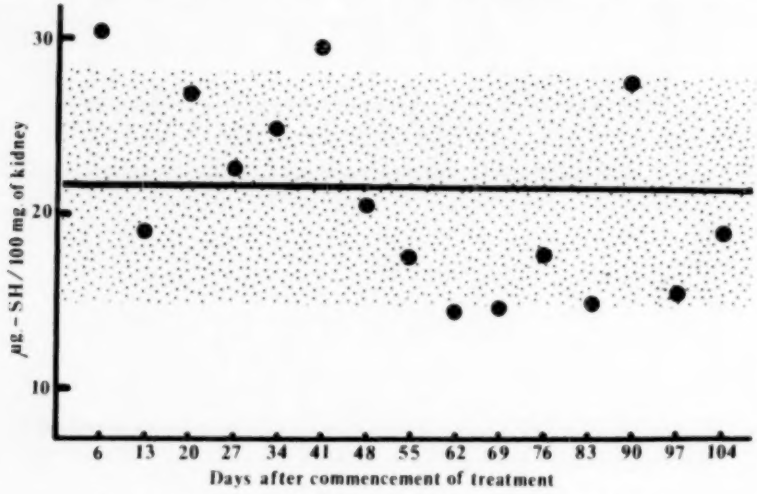

FIG. 5.—The effects of feeding 2.A.A.F. on rat kidney –SH levels.

–SH during the first six weeks of the experiment and then a fall to below the mean control value.

As a tissue which is not susceptible to tumour induction by 2.A.A.F. we have taken cardiac muscle (from the ventricles) of both control and treated animals The results are given in Fig. 6. The picture here rather resembles that obtained with the kidney.

p-*Dimethylaminoazobenzene* (*dimethyl yellow*).—This agent is a potent hepatocarcinogen in rats on a poor diet. Fifty female Wistar rats aged twelve weeks were divided into two groups and given a diet of rice and water supplemented with carrots twice weekly. After ten days on this diet one group had dimethyl yellow in olive oil added to their rice at the rate of 0·6 per cent of the diet. The control group had a similar amount of olive oil added to their rice. At weekly intervals commencing six days after treatment began, the liver –SH level was measured on a rat from each group. The results are shown in Fig. 7. As the control figures varied widely—possibly as a result of dietary effects—no mean

figure has been calculated, but each pair of results has been plotted. Between 27 and 48 days after commencement of treatment the experimental figures were above the controls, thereafter the position was reversed.

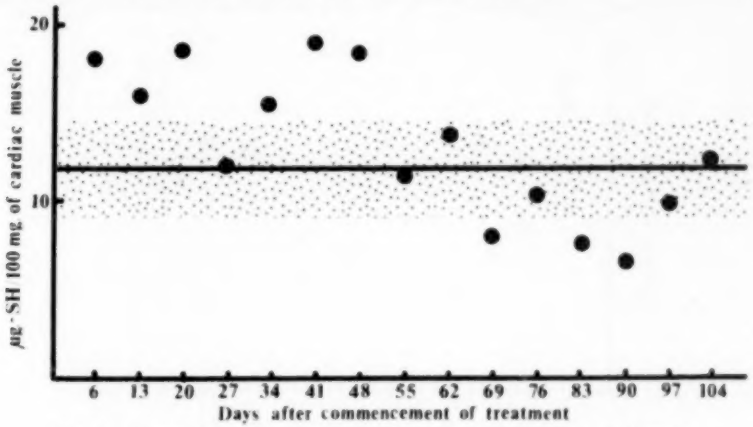

FIG. 6.—The effects of feeding 2.A.A.F. on rat cardiac muscle –SH levels.

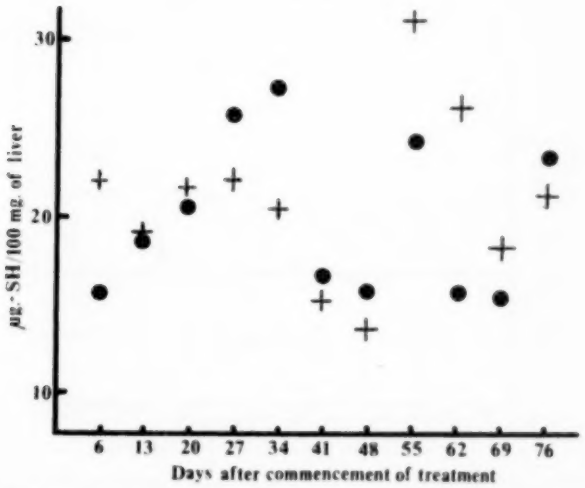

FIG. 7.—The effects on liver –SH values of feeding *p*-dimethylaminoazobenzene to rats on a poor diet. Control values are shown as crosses.

–SH measurements have also been made on the kidneys of the same rats. The results are given in Fig. 8. Here again the individual readings have been plotted, although the fluctuations in the control levels are only minor in nature. There appears to be little distinction between the two sets of figures.

Ethyl carbamate (*urethane*).—Forty female Strong A mice aged 10 weeks were divided into two groups of 20 each. Both received the normal pellet diet as food whilst one was given tap water to drink the other received a 0·1 per cent solution of urethane in tap water. At weekly intervals commencing 6 days after

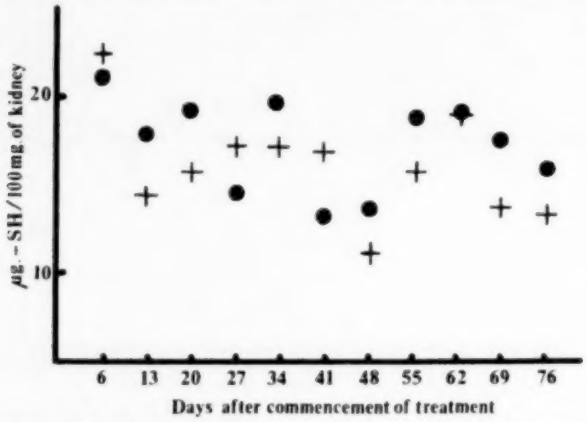

FIG. 8.—The effects of feeding dimethylaminoazobenzene on rat kidney –SH levels. Control values are shown as crosses.

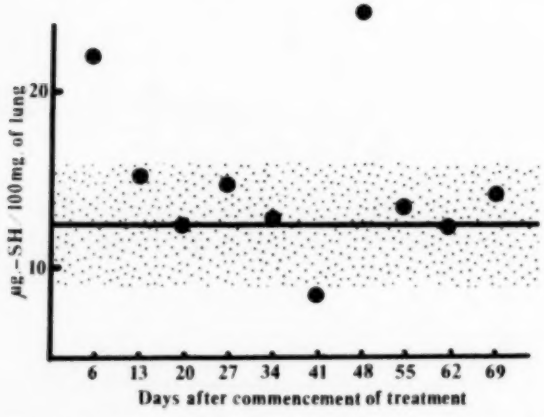

FIG. 9.—The effects of drinking 0·1 per cent urethane on mouse lung –SH levels.

the transfer to urethane one mouse of each group was taken and the –SH values for the lung tissue were measured. The results are shown in Fig. 9, from which it appears that the experimental animals show a tendency to an elevated –SH level, as compared with the controls.

It was found by Haran and Berenblum (1956) that mice which had received urethane systemically did not normally produce skin tumours, but that tumours

would occur following further treatment with croton oil. We have, therefore, estimated the –SH levels in the skins of mice used in the previous experiment. The results are given in Fig. 10, from which it is seen that there is a persistent decline in –SH values as compared with the control values.

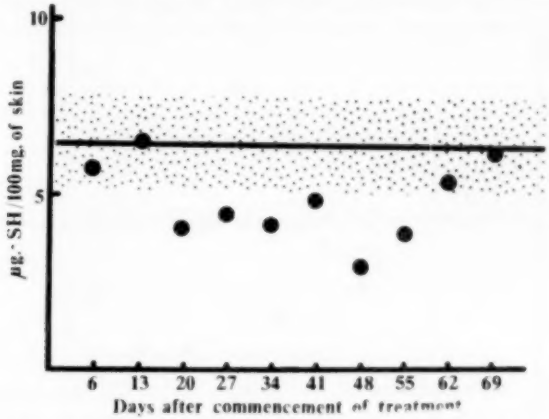

FIG. 10. The effects of drinking 0·1 per cent urethane on mouse skin –SH levels.

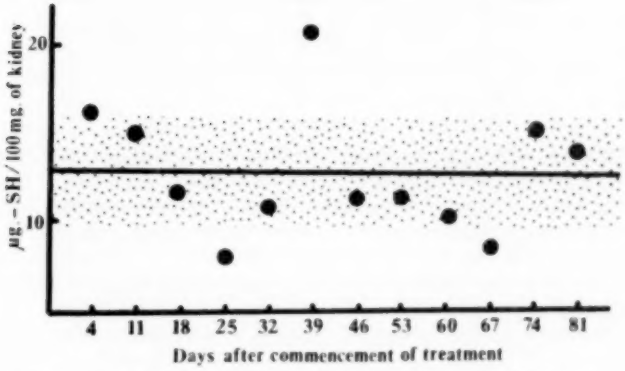

FIG. 11.—The effects of stilboestrol on hamster kidney –SH levels.

Stilboestrol.—Twenty male hamsters aged eight weeks each received a subcutaneous implant of one 15 mg. pellet of stilboestrol. A similar untreated group was kept as controls. Starting four days after the implantations one of each group was killed weekly and the kidney –SH levels were measured. The results are shown in Fig. 11. Generally, the experimental animals have tended to show a slightly lower –SH value than the controls. This experiment, however, has not proved very satisfactory as the absorption of the pellets has been very variable. It was noticed that in the case of the two high readings (39 and 74 days) the

implants had almost disappeared, but in some of the other animals their appearance was as when put in.

Measurements of –SH have also been made on the liver tissue ; the results being given in Fig. 12. Here again the experimental figures fall rather below the control level.

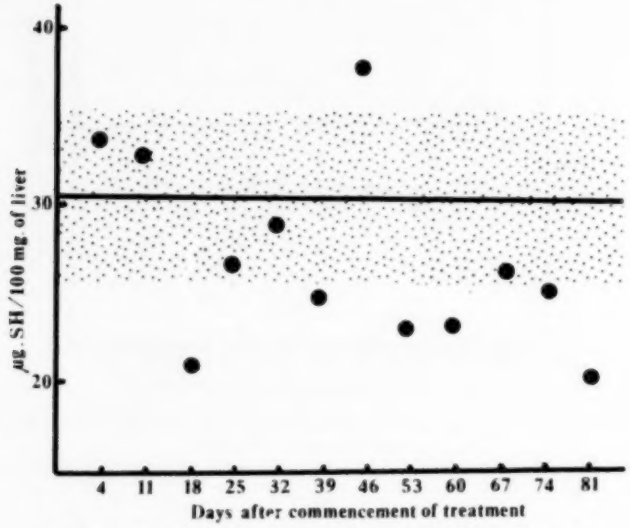

FIG. 12.—The effects of stilboestrol on hamster liver –SH levels.

The Source of Elevated –SH Levels

Reference was made earlier to the finding by Boyland and Mawson (1938) of elevated liver glutathione levels after treatment with 3 : 4 : 5 : 6 dibenzocarbazole. It is obviously of interest to know whether elevated levels found in some instances in the present work are the consequence of a change in glutathione levels or also represent changes in protein –SH levels. Glutathione estimations have been done in a few cases during the experiments with N.2 fluoroenylacetamide and *p*-dimethylaminoazobenzene.

Table I records figures obtained during experiments with 2.A.A.F. The

TABLE I.—*The Distribution of –SH in μg./100 mg. of Liver of Control Rats and Rats Fed 2.A.A.F.*

	Control			2.A.A.F.		
	Total –SH	Glutathione –SH	Protein –SH	Total –SH	Glutathione –SH	Protein –SH
	26·4	12·5	13·9	29·9	13·2	16·7
	24·1	7·6	16·5	30·6	12·1	18·5
	27·5	10·5	17·0	34·6	10·3	24·3
	25·2	14·4	10·8	27·3	16·9	10·4
	26·0	14·2	11·8	29·6	15·5	14·1
Mean	25·8	11·8	14·0	30·4	13·6	16·8

figures are very limited in number but do suggest that both glutathione and protein –SH have been affected.

Table II records similar figures obtained with rats fed *p*-dimethylaminoazobenzene together with a rice diet. Here, because of the limited figures available and the wide fluctuations in levels conclusions are difficult, but certainly an impression is gained that both the glutathione and protein levels are subject to increase.

TABLE II.—*The Distribution of –SH in μg./100 mg. of Liver from Control Rats and Rats Fed* p*-dimethylaminobenzene*

Control			*p*-Dimethylaminoazobenzene		
Total –SH	Glutathione –SH	Protein –SH	Total –SH	Glutathione –SH	Protein –SH
20·4	5·3	14·9	27·1	9·0	18·1
15·5	3·1	12·4	16·6	5·8	10·8
13·5	5·9	7·6	15·7	7·5	8·2

DISCUSSION

The work recorded above is part of an extensive programme designed to investigate the role of tissue –SH groups in the carcinogenic process. In view of this and the paucity of data in the field as a whole, discussion here is limited to certain points of immediate interest in relation to the results obtained.

The figures obtained indicate that 3 : 4 benzopyrene, N.2 fluoroenylacetamide and urethane are capable of inducing an elevation of –SH levels in tissues upon which they exert their carcinogenic response. Suggestions of a similar effect with *p*-dimethylaminoazobenzene and stilboestrol were also obtained. On the other hand, the agents used have only shown one example of a non-susceptible tissue –SH level being affected. That is the case of urethane and mouse skin. There the evidence would imply deletion of sulphydryl as was previously found by Calcutt *et al.* (1959) for the case of anthracene and liver tissue. In this particular case it is known that mercapturate formation plays a part in the metabolism of anthracene, but there is no evidence available of mercapturate formation after treatment with urethane. However, it may or may not be significant that Bieckert (1951) has found an unknown reducing substance in the urine of man after urethane treatment.

The slight evidence available indicates that where –SH levels have risen the increase is in both glutathione and protein –SH content. Boyland and Mawson (1938) found increased liver glutathione after treatment with the hepatocarcinogen 3 : 4 : 5 : 6 dibenzocarbazole but did not measure protein –SH. Crabtree (1946) measured the glutathione content of mouse skin after treatment with various polycyclic hydrocarbons. Falls in level were found with the known mercapturate producing agents—anthracene and phenanthrene ; whilst 1 : 2 : 5 : 6 dibenzanthracene and 3 : 4 benzopyrene had no effect. As these experiments were conducted over a very limited time period (up to 4 hours) too much weight should not be attached to these findings.

Where increases in –SH content have occurred another problem is posed in the question as to the origin of this new reactive material. In the case of glutathione it could arise at the expense of oxidised glutathione by reduction, although

almost certainly not by the direct chemical action of the carcinogenic chemicals used. Alternatively it would appear to be new formation of glutathione. In the case of the cellular protein the increased –SH could be derived from either one or a combination of the following: breakage of existing S–S bonds to give –SH; an actual increase in the cell protein content or an apparent increase in protein content as the result of loss of water. In this connection it may be pertinent to notice that Barer and Joseph (1960) found that the lymphocytes from mice which had been treated with X-rays or Chlorambucil (both of which may be regarded as carcinogenic agents) showed an increased cytoplasmic protein content over periods of up to 28 days.

The point still remains as to whether –SH levels are concerned in the induction of tumours. Certainly, the present work is suggestive of chemical carcinogens being able to bring about a rise in levels in susceptible tissues but not in non-susceptible tissues. Alongside this must be placed the experience of Crabtree (1944, 1946) that mercapturate forming agents—bromobenzene, anthracene and phenanthrene—act as inhibitors to the induction of skin tumours by polycyclic hydrocarbons. Additionally, Crabtree (1945) has also found that dibasic acids, such as citraconic and malonic acids, inhibit –SH groups and also inhibit skin tumour induction. Crabtree considered the inhibition of tumour induction in terms of removal of –SH groups necessary for combination with the carcinogen, but now it may appear more relevant to consider the matter in terms of prevention of a rise in tissue –SH.

The previous paragraphs have raised many questions which require further experimental work for their solution. It is hoped to be able to proffer answers to some of these problems in some future papers.

SUMMARY

3 : 4 Benzopyrene has been found to induce an increase in the –SH content of mouse skin, but not in that of mouse liver or kidney.

N.2 fluoroenylacetamide causes an increase in liver –SH levels in rats. Effects on rat kidney and heart muscle were ill defined but suggestive of a rise in levels.

p-Dimethylaminoazobenzene caused a slight increase in the liver –SH levels of rats (as compared with controls) over a limited period. No effect was detected in kidneys.

Urethane was found to increase mouse lung –SH levels but to depress mouse skin levels.

Stilboestrol gave some indications of elevating kidney –SH levels in hamsters but caused a slight fall in liver levels in the same animals. These findings have been briefly discussed.

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REFERENCES

BARER, R. AND JOSEPH, S.—(1960) *Exp. Cell. Res.*, **19**, 51.

BIECKERT, A.—(1951) *Z. ges. exp. Med.*, **117**, 10, quoted by Williams, R. T. (1959) "Detoxication Mechanisms". London (Chapman and Hall).

BOYLAND, E. AND MAWSON, ELINOR, H.—(1938) *Biochem. J.*, **32**, 1460.

CALCUTT, G. AND DOXEY, D.—(1959) *Exp. Cell. Res.*, **17**, 542.
Idem, DOXEY, D. AND COATES, JOAN.—(1959) *Brit. J. Cancer*, **13**, 711.
Idem AND POWELL, A. K.—(1947) *Ibid.*, **1**, 323.
CRABTREE, H. G.—(1944) *Cancer Res.*, **4**, 688.—(1945) *Ibid.*, **5**, 346.—(1946) *Ibid.*, **6**, 553.
DI PAOLO, J. A. AND NIEDBALA, T. F.—(1957) *Proc. Soc. exp. Biol. N.Y.*, **96**, 255.
HARAN, NECHAMA AND BERENBLUM, I.—(1956) *Brit. J. Cancer*, **10**, 57.
HARTWELL, J. L.—(1951) "Survey of compounds which have been tested for carcinogenic activity." 2nd Ed. Bethesda (National Cancer Institute).
SHUBIK, P. AND HARTWELL, J. L.—(1957) "Survey of compounds which have been tested for carcinogenic activity." 1st Supplement. Bethesda (National Cancer Institute).
STOCK, J. T.—(1958) *Analyst*, **83**, 56.
WEIGERT, F. AND MOTTRAM, J. C.—(1946) *Cancer Res.*, **6**, 109.

RELATIVE METABOLIC ACTIVITIES OF HISTONES IN TUMOURS AND LIVER

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VERY little is known about the biosynthesis of histones in either normal or cancerous tissue. As major constituents of the chromosomes, it might be expected that their synthesis will be closely connected with and possibly controlled by DNA synthesis. As the rate of DNA synthesis is determined by the rate of cell division, it follows that histone synthesis may similarly differ in actively dividing and non-dividing tissues.

Comparisons between tissues using radioactive amino acids are complicated by unknown factors such as the rate of transport of the labelled compound to the site of synthesis and the dilution by the non-radioactive amino-acid pool (Loftfield and Harris, 1956). Correct information on these factors may be difficult to obtain but comparisons of limited significance can be made if the activity of the cytoplasmic proteins is used as a standard of reference. Such comparisons might well show a marked difference between actively dividing tissues and non-dividing cells.

An experiment of this kind was carried out by Rotherham *et al.* (1957) who compared the partition of glycine-^{14}C between nuclear and cytoplasmic proteins of rat hepatoma and normal rat liver. They found that 20 hours after injection, the specific activities of nuclear histones were considerably less than those of the cytoplasmic proteins in both cases, although the ratio of labelling of histone and cytoplasmic protein (H/C) was greater in the case of tumour (0·5) than for normal liver (0·15).

Busch, Davis and Anderson (1958) and Busch *et al.* (1959) made similar comparisons between the rates of uptake of various amino-acids into the histones of Walker and Jensen rat tumours and other normal rat tissues, the corresponding uptake into cytoplasmic proteins being also obtained. Within 1 hour of injection the specific activity of the tumour histones was comparable with the activity of the most active cytoplasmic fraction of the tumour (H/C = 1·2). In normal tissues on the other hand the reverse was generally true (H/C = 0·6).

In the original paper of Busch *et al.* (1958) the histone was obtained as a precipitate on neutralising an acid extract of nuclei. It has been shown by Davison (1957), both with tumours and rat liver, that this procedure gives a complex of histone with an acidic protein. In a subsequent paper (Busch *et al.*, 1959) precipitation of dialysed nuclear extract with acetone and ammonia was employed which should give a better product.

In this laboratory we have developed methods for preparation of histone which are known to give a product with acceptable analytical properties, either by extraction of the whole histone from isolated deoxynucleoprotein or by isolation

of a well characterised histone fraction by extraction in the presence of 70 per cent alcohol (Johns *et al.*, 1960). The latter method is so specific that in the case of calf thymus, no initial separation of the nuclei is necessary. We have therefore repeated some of the work with our own methods to ensure that the effects observed are not due to non-basic impurities.

EXPERIMENTAL

Radioactive *l*-lysine-U-^{14}C (specific activity 7·5 mC/mM) was obtained from the Radiochemical Centre, Amersham. Rats were of CB strain, average weight 250 g. and mice were CB stock, average weight 30 g. The animals were fed until 5 hours before the experiments. Injections were intraperitoneal and the animals were killed 1 hour after injection. Counting was at near infinite thickness on 1 cm^2. or 0·25 cm.^{2} planchettes, a small correction being applied for the actual weight of protein on the planchette. An end window counter was used, the background being 10–11 c.p.m. measured over 30 or 60 minutes. Samples were counted for 10 minutes each. Amino acid analyses were by the method of Phillips and Johns (1959).

Histones from rats carrying the Walker tumour (Method A)

Rats, having received an implant of the Walker tumour 7 days previously, were injected with 5 μc. lysine each. After killing the animals' livers and tumours were rapidly removed and separately frozen on solid CO_2 blocks, necrotic regions of the tumour being discarded. Frozen tissues were stored at — 20° C. and used within 3 hours.

The frozen tissues (35 g.) were each placed in enough ice cold 0·9% w/v NaCl solution (pH 7) to bring the total volume to 200 ml. After standing for a few minutes the partially thawed tissues were homogenised for two minutes in an MSE homogeniser running at maximum speed (14,000 r.p.m.). The nucleoprotein fractions were obtained by centrifuging at 1500 **g** for 30 minutes, the supernatant liquid being retained for isolation of cytoplasmic proteins. Nucleoprotein was washed twice in 0·9 per cent NaCl by repeating the homogenisation and centrifuging. The washed nucleoproteins were each extracted overnight with 60 ml. 0·25 N-H_2SO_4 at 4° by rocking in vessels containing glass beads. The extract was clarified in a Spinco model L refrigerated ultracentrifuge (20,000 **g**, 30 minutes) and adjusted to pH 3·8 by addition of solid barium hydroxide. The barium sulphate precipitate was removed by centrifuging (2000 **g**, 30 minutes). The histone was then obtained from the remaining solution by adjustment to pH 10 and addition of 3 vol. of acetone as described by Davison (1957).

Histones from mice carrying the Crocker tumour (Method A)

The mice received an implant of the Crocker tumour 9 days before injection with 1 μc lysine each. No necrotic areas were found in the tumours and 25 g. of tissue were treated as described for the Walker tumour.

Arginine-rich histone from rat liver (Method B)

75 g. of liver tissue from rats injected with 5 μc lysine each, were frozen by dropping into an acetone-solid CO_2 bath. The tissue was used after 2 hours'

storage. It was partially thawed in 600 ml. ice cold saline (0·9 per cent w/v, pH 3·5) and homogenised in a Waring blendor run at full speed for 2 minutes. The nucleoprotein was separated by centrifuging for 30 minutes at 800 g., the supernatant liquid being retained. The nucleoprotein was washed once in acidific saline and twice in 70 per cent ethanol. The nucleoprotein was then extracted overnight with 200 ml. 0·25 N—HCl in 70 per cent ethanol in a ball mill.

The histone contained in the acid extract was clarified (1500 ***g***, 30 minutes) at room temperature. The insoluble residue was extracted with a further 200 ml. acid ethanol. The arginine-rich histone was then precipitated from the combined extracts by dialysis against several changes of absolute alcohol, the precipitate being dried with acetone.

Arginine-rich histone from mouse Ehrlich ascites (Method B)

Mice had received an injection of Ehrlich ascites cells 7 days before injection with 5 μc. amino acid each. The ascites fluid was obtained within a few minutes after killing the animals and the cells were washed twice with 0·9 per cent NaCl using a centrifuge run at low speed. The washed cells were frozen in liquid nitrogen and homogenised after partial thawing with an equal volume of 0·14 M NaCl containing 0·01 M sodium citrate (pH 7) in a glass homogeniser with a Teflon plunger. The nuclei were isolated by centrifuging at 1500 ***g*** for 10 minutes and washed twice with the saline citrate mixture. After washing twice with 70 per cent alcohol the nuclei were extracted overnight with 0·25 N—HCl in 70 per cent alcohol by shaking with glass beads. The arginine-rich histone was precipitated as for rat liver.

Cytoplasmic proteins

After removal of the nuclei or nucleoprotein, a sample of the whole cytoplasmic protein was precipitated by addition of an equal volume of 10 per cent trichloracetic acid and heating to 90° C. for 10 minutes. The coagulated protein was washed with 5 per cent trichloracetic acid, alcohol, acetone, ether and then dried.

RESULTS

Table I shows the specific activities recorded for the various histones and the corresponding cytoplasmic fractions prepared in the present work. Values of H/C are given for each tissue together with the method of preparation.

TABLE I.—*Incorporation of l-Lysine into Histone and Cytoplasmic Protein of Various Tissues**

Tissue	Method of preparation of histone	Cytoplasmic protein (C)	Histone (H)	H/C
1. Rat liver	A	171	120	0·70
2. Rat liver	B	115	111	0·95
3. Rat Walker tumour	A	92	135	1·46
4. Mouse liver	A	206	112	0·54
5. Mouse tumour	A	129	144	1·12
6. Ehrlich ascites tumour in mouse	B	1270	1220	0·96

* Values given are counts per minute/cm^2 at infinite thickness.

In Table II are given amino acid analyses for histones prepared by the various methods described, together with some analyses of the cytoplasmic fractions. The histone preparations contain 23–25 per cent of basic amino acids, and 13–15 per cent acidic amino acids, which is within the range typical of histones (7). The cytoplasmic protein fractions contain 15 per cent of basic amino acids and about 21 per cent of acidic amino acids.

TABLE II.—*Amino Acid Analyses Given as Moles of Amino Acid per* 100 *Residues Determined*

	Histones			Cytoplasmic proteins	
Amino acid	Mouse liver (Method A)	Mouse Crocker tumour (Method A)	Rat liver (Method B)	Rat liver	Mouse tumour
Asp	5·9	4·8	6·8	8·4	11·6
Glut	7·6	8·0	8·3	12·1	10·6
Gly	9·0	8·7	6·8	8·4	6·9
Ala	12·6	12·4	13·3	8·7	8·9
Val	7·7	7·5	5·6	7·9	6·9
Leu	11·1	12·2	12·7	14·9	13·4
Se	5·9	5·9	5·3	6·3	6·5
Thr	6·3	6·3	6·3	5·5	5·5
Phe	1·7	1·9	2·8	4·5	3·8
Tyr	1·7	2·2	2·0	2·4	4·3
Pro	5·2	4·7	6·8	5·6	5·2
His	2·3	2·3	2·7	1·8	1·9
Lys	14·0	13·9	11·4	8·1	8·3
Arg	8·9	9·2	9·2	5·6	5·2
Met	—	—	—	—	0·9

DISCUSSION

The results are in agreement with the finding of Busch *et al.* (1958, 1959) in that H/C is appreciably greater in the tumours than in normal tissues of the same animal. However, leaving out of consideration the Ehrlich ascites tumour, which is not comparable in that the amino acid was applied directly within the tumour, it can be seen that the activities of the cytoplasmic proteins from liver and tumour of similar animals, differ more than those of the histones. The differences of H/C are therefore to a considerable extent due to variations of C and it cannot be concluded with certainty from these results that the tumour histones are markedly more active metabolically than the histones of comparable normal tissues.

In fact a possible explanation of these results is that the tissue cytoplasm is markedly defective in its ability to incorporate amino acids as compared with the normal tissues of the same animal. The experiment with the mouse Ehrlich ascites tumour is a special case and a direct comparison of the actual amounts incorporated in this and the other mouse tissues is meaningless. The value of H/C is not greatly different, however, from that of the other mouse tumour and is markedly greater than H/C for mouse liver. This tissue is therefore not in

contradiction to the other cases. The actual levels of labelling in the different cases (except the ascites tumour) are surprisingly similar.

In conclusion we may comment on the fact that the degree of labelling of the histones is in all cases comparable with (and in tumours greater than) that of the cytoplasmic proteins. A moderately high degree of metabolic activity of histones was observed in early experiments by Daly, Allfrey and Mirsky (1952), Brunish and Luck (1952) and Smellie, McIndoe and Davidson (1953). In these experiments the degree of labelling of the histone fraction was usually 1/3 that of the cytoplasmic proteins and although the histone was often the least labelled fraction the activity was higher than could be accounted for as due to the rate of synthesis required for replication of chromosomes in cell division.

The activity observed in the cytoplasmic proteins will depend on the mode of preparation and will vary with the amount of inactive structural protein included. Even in the cytoplasmic particles there exist proteins with very different rates of incorporation (Cohn and Butler, 1958) and it may well be that the activities of the cytoplasmic proteins quoted are well below those of the most active fractions. Nevertheless, the relative incorporation in tumour and liver found here can hardly be explained except on the assumption that turnover or synthesis of histones occurs besides that necessary for cell division.

A number of authors (e.g. Stevens, Daoust and Leblond, 1953) have shown that ^{32}P incorporation into DNA of various normal tissues is proportional to the mitotic rate. For a 200 g. rat, Stevens *et al.* estimated that 0·71 per cent of liver cells divide per day, whereas for a rapidly growing tumour almost all the cells may divide once in 24 hours and some may divide twice. If the incorporation of histone were proportional to the mitotic rate, a difference of 100-fold would be expected between the incorporation into the histone of tumour and liver. The difference found is very much less than this, and from the results of Greenberg and Sassenrath (1955) the distribution of amino acids after intraperitoneal injection appears to be too uniform to introduce a large deviation from the expected difference of this order. Therefore the rate of incorporation into liver histone is greater than could be expected for the synthesis of chromosome histone necessary to maintain the mitotic cycle, and we must conclude that histones undergo an appreciable amount of turnover or additional synthesis.

SUMMARY

1. The incorporation of *l*-lysine into histones prepared by two different methods and into cytoplasmic proteins of rat and mouse tumours as well as rat and mouse liver has been studied under comparable conditions.

2. The ratio of the specific activity of histone (H) to that of the cytoplasmic proteins (C) of the same cells is greater for the tumours than for the liver of the same animal. However, the differences in these ratios appear to be due to differences of C to an even greater extent than to differences of H and it is possible that in tumour cells the rate of incorporation of amino acids into the cytoplasmic proteins is abnormally low.

3. The differences of rates of incorporation in the tumours and similar normal tissues do not appear to be related to mitotic rates. It is necessary to conclude that a significant turnover or synthesis of histone occurs in resting cells.

We are indebted to Miss P. Simson for carrying out the amino acid analyses given; to Messrs. B. Mitchley and C. Smith for implanting the tumours.

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REFERENCES

Brunish, R. and Luck, J. M.—(1952) *J. biol. Chem.* **198**, 621.
Busch, H., Davis, J. R. and Anderson, D. C.—(1958) *Cancer Res.*, **18**, 91?
Idem, Davis, J. R., Honig, G. R., Anderson, D. C., Nair, P. V. and Nyhan, W. L. —(1959) *Ibid.*, **19**, 1030.
Cohn, P. and Butler, J. A. V.—(1958) *Biochem. J.*, **70**, 254.
Daly, M. M., Allfrey, V. G. and Mirsky, A. E.—(1952) *J. gen. Physiol.*, **36**, 173.
Davidson, P. F.—(1957) *Biochem. J.*, **66**, 708.
Greenberg, D. M. and Sassenrath, E. N.—(1955) *Cancer Res.*, **15**, 620.
Johns, E. W., Phillips, D. M. P., Simson, P. and Butler, J. A. V.—(1960) *Biochem. J.*, **77**, 631.
Loftfield, R. B. and Harris, A.—(1956) *J. biol. Chem.*, **219**, 151.
Phillips, D. M. P. and Johns, E. W.—(1959) *Biochem. J.*, **72**, 538.
Rotherham, J., Irvin, J. C., Irvin, E. M. and Holbrook, D. J.—(1957) *Proc. Soc. exp. Biol., N.Y.*, **96**, 21.
Smellie, R. M. S., McIndoe, V. M. and Davidson, J. N.—(1953) *Biochim. biophys. Acta*, **11**, 559.
Stevens, C. E., Daoust, R. and Leblond, C. P.—(1953) *J. biol. Chem.*, **202**, 177.

SURVEY OF PAPERS

MUIR and KIRK discuss the evidence for regarding tobacco as the carcinogen concerned in cancer of the mouth due to betel chewing, and report some experiments in which mice were painted daily with an extract of a typical Singapore betel/tobacco quid (p. 597).

FERRARI and KREYBERG discuss the differences in incidence and histological types of lung cancer in Venice and Norway (p. 609).

THACKRAY and LUCAS describe and illustrate the histological variations seen in cylindromas of mucous gland origin (p. 612).

BREW and JACKSON find that involvement of the ovaries is a common and conspicuous feature of lymphosarcoma in young African girls in Nigeria (p. 621).

GREENING, RAMSAY, STEVENSON, BOYLAND, RIGBY-JONES and GODSMARK report the results of treating 100 patients with advanced breast cancer by interstitial irradiation of the pituitary. They consider that this method of treatment is well worth continuing and that it might be of value in other conditions (p. 627).

HIRCHINSON and HILL describe the effects of monocrotaline on human embryo liver in tissue culture. This senecio alkaloid appears to induce *in vitro* amitotic division and possibly neoplastic changes in embryonic liver cells (p. 637).

GHADIALLY, ILLMAN and BARKER find that single or repeated trauma does not produce malignant change in carcinogen induced melanotic tumours of hamsters (p. 647).

CLEMO and MILLER describe experiments in which a definite tumour-promoting effect was observed when the neutral fraction from cigarette smoke was applied to mice after an initiating dose of a fraction from city smoke (p. 651).

HOWELL finds that hormonal factors such as ovariectomy, continued breeding and lactation prevent the induction of breast tumours in female rats' skin painted with oily solutions of 9 : 10-dimethyl-1 : 2-benzanthracene. Male rats similarly treated do not develop breast tumours, neither do castrated males (p. 657).

BIANCIFIORI and CASCHERA report the successful conversion of a mucoid adenocarcinoma of the breast arising spontaneously in a female mouse of the Balb/C+ strain to an ascites tumour by repeated intraperitoneal transfer (p. 668).

NEGRONI and CHESTERMAN show that the cells of hamster kidney tumours induced by Mill Hill polyoma virus do not produce virus (p. 672).

STOKER has studied the oncogenic activity of the Toronto strain of polyoma virus in hamsters. Foci of tumour cells appear in the kidneys of new born hamsters 4 days after inoculation ; counts of these foci are roughly proportional to the virus dose, suggesting that each focus is initiated by a single virus cell interaction (p. 679).

BITENSKY, BALDWIN and CHAYEN find no evidence that a single injection of 4-dimethylaminostilbene is followed by either binding of the carcinogen to protein or permanent depression in succinic dehydrogenase activity in the liver cells (p. 690).

BITENSKY, BALDWIN and CHAYEN have studied the immediate response of rat liver to the carcinogen 4-dimethylaminostilbene by histochemical methods. Their findings suggest that the carcinogen, or a metabolite, frees the bonds between lipids and proteins, and causes some type of phase inversion. In addition there is morphological damage to the mitochondria of the liver cells (p. 696).

Lyons and Spence have estimated the relative amounts of free radicals to which cigarette smokers and non-smokers are liable to be exposed. They find that the radicals in diesel soot and domestic chimney soot are extremely stable and insoluble, and it seems unlikely that they play any part in carcinogenesis. Other active free radicals produced in polluted urban and industrial atmospheres are briefly discussed and their possible danger for man indicated (p. 703).

Houghton and Martin have examined abnormal globulins of varying mobilities from patients with myelomatosis. The ultracentrifugal and immunochemical analyses suggest that these globulins show a progressive deviation from the normal rather than falling into well defined groups (p. 709).

Trevan and Roberts record the behaviour *in vitro* of cells of an ascites subline of a naturally occurring murine epithelioma at and about the time of their division (p. 716).

Trevan and Roberts describe sheet formation by the cells of a murine ascites epithelioma when cultured on a glass surface, and discuss the factors affecting the stability of these sheets (p. 724).

Siegel, Sachs and Graig have studied the ^{35}S levels in the blood and plasma of patients with multiple myeloma and with other malignant diseases following intravenous administration. The myeloma patients were found to have longer initial and late half-disappearance times, suggesting the hyperglobulinaemia of this disease may result from a slower catabolism of myeloma globulin (p. 730).

Neish and Rylett find that single intraperitoneal injections of powerful hepatocarcinogens into male albino rats cause a marked accumulation of phosphoethanolamine in the livers (p. 737).

Calcutt, Doxey and Coates report measurements of the —SH levels in susceptible and non-susceptible tissues of mice, rats and hamsters at intervals after treatment with chemical carcinogens. They find that, in general, the carcinogens tested induce an elevation of —SH levels in the tissues on which they exert their carcinogenic response (p. 746).

Butler and Laurence have studied the rates of incorporation of *l*-lysine into histones and into cytoplasmic proteins of rat and mouse tumours as well as rat and mouse liver. They discuss the possible significance of the differences found (p. 758).

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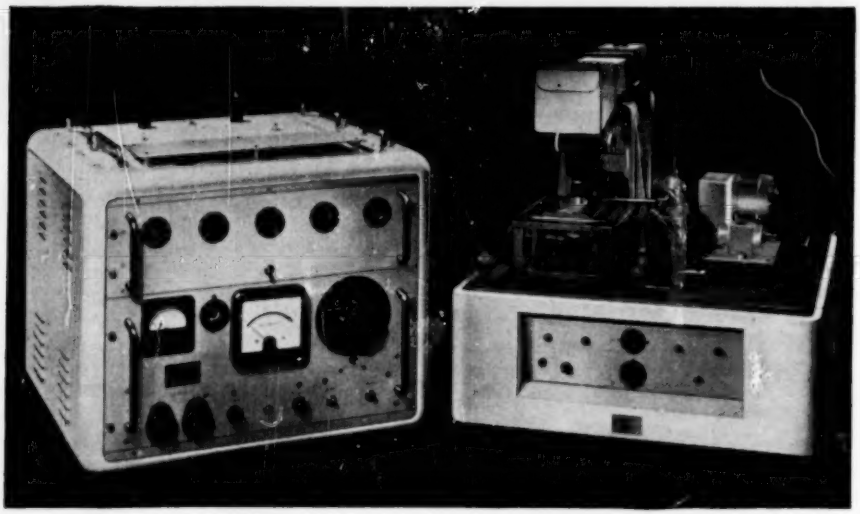

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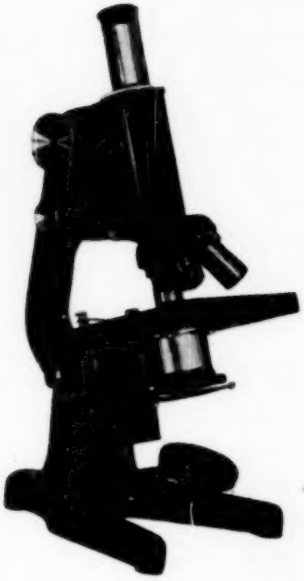

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